The plastid genome and its expression in potato (Solanum tuberosum L.)

Conover, Joanne Carol

Award date:
1986

Awarding institution:
University of Bath

THE PLASTID GENOME AND ITS EXPRESSION IN POTATO (*SOLANUM TUBEROSUM L*)

Submitted by Joanne Carol Conover
for the degree of PhD
of the University of Bath
1986

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

ACKNOWLEDGEMENTS

I would like to thank my supervisors, Dr. Janet A. Pryke and Prof. P.D.J. Weitzman, for their encouragement, support and advice throughout this work. Special thanks go to all the members of the lab for their helpful discussion, to Mr. Simon Hankins for his continued encouragement and help, and to Mrs. Paula Keilthy for typing this thesis. I would also like to express my gratitude to the statistics staff of The Weed Research Organisation, Oxford, for the use of their computer facilities.

I gratefully acknowledge the British Government for a two year Overseas Research Studentship.

To my family

SUMMARY

The greening of potato tubers (*Solanum tuberosum L*) on exposure to light provides an ideal system with which to study amyloplast to chloroplast transformation. Some molecular aspects of the light-induced transformation are described in this thesis.

The peripheral cells (outer parenchyma and periderm tissue layers) of tuber tissue were selected for this study due to (1) chlorophyll synthesis occurring in mainly the first 3 mm of tuber tissue, and (2) the relative uniformity in cell size of this tissue.

The ploidy of nuclei from the peripheral cells was determined in tubers exposed to varying lengths of illumination. The average nuclear ploidy in normal white potatoes was found to be approximately 23C. Similar values were also found for tubers exposed to 8 and 12 days of illumination. However, tubers exposed to only 4 days of illumination were found to have an average ploidy level of 15C. This decreased ploidy level is thought to be the result of synchronised cell division, which is possibly light-induced.

Restriction endonuclease digestion pattern analysis showed that different species of potato have similar plastid DNAs (pt DNAs). A restriction endonuclease cleavage site map is proposed for potato based on the well-documented cleavage site map of the closely related tobacco pt DNA.

Dot blot hybridisation was used to quantify the levels of pt DNA in a total DNA population. Expressed as a percentage of the total DNA, the level of pt DNA in non-illuminated tuber tissue (15%)

was more than found in leaf tissue (7%), while tubers exposed to light (4 and 8 days of illumination) showed the highest pt DNA levels (22%).

Analysis of pt rRNA levels in white and green tuber tissue were achieved by polyacrylamide gel electrophoresis (PAGE) and dot blot hybridisation. Very low levels of pt rRNA were detected in white tuber tissue. However, greening of the tuber tissue (4 days of illumination) resulted in increased pt rRNA levels to ¼ that found in leaf tissue. Further greening showed a slight decrease in pt rRNA levels.

Attempts were also made to determine levels of mRNAs for the genes: atpA, atpB, atpE, petA, and rbcL, in illuminated and non-illuminated tuber tissues.

ABBREVIATIONS

Ap^r	-	ampicillin resistance
ATP	-	adenosine triphosphate
atpA, B or E	-	the gene for the α, β or ε subunits of the extrinsic component CF_1 of ATP synthase
BSA	-	bovine serum albumin
$CHCl_3$	-	chloroform
cpm	-	counts per minute
ct DNA or RNA	-	chloroplast deoxyribonucleic acid or ribonucleic acid
CTP	-	cytidine 5'-triphosphate
DTT	-	dithiothreitol
DNA	-	deoxyribonucleic acid
EDTA	-	ethylenediaminetetra-acetic acid
IPTG	-	isopropylthio-β-D-galactoside
kbp	-	kilo base pairs
kd	-	kilo dalton
kPa	-	kilo pascal
mRNA	-	messenger ribonucleic acid
mt DNA	-	mitochondrial DNA
PAGE	-	polyacrylamide gel electrophoresis
PCA	-	perchloric acid
petA	-	the gene for cytochrome f
PMSF	-	phenylmethyl sulphonyl fluoride
PPO	-	1-phenyl-4-phenyloxazole
pt DNA or RNA	-	plastid deoxyribonucleic acid or ribonucleic acid
PVP	-	polyvinylpyrrolidone
rbcL	-	the gene for the large subunit of ribulose 1,5-bisphosphate carboxylase
RNA	-	ribonucleic acid
rRNA	-	ribosomal ribonucleic acid
RuBPcarboxylase	-	ribulose 1,5-bisphosphate carboxylase

TCA	-	trichloroacetic acid
Tc^r	-	tetracycline resistance
TEMED	-	*N,N,N',N'*-tetramethyl-ethylendiamine
tris	-	tris(hydroxymethyl)methylamine
tRNA	-	transfer RNA
TTP	-	thymidine 5'-triphosphate
SDS	-	sodium dodecyl sulphate
var	-	variety
x-gal	-	5-bromo-4-chloro-3-inddyl-β-*D*-galactoside

CONTENTS

CONTENTS (Continued)

CONTENTS (Continued)

CONTENTS (Continued)

CONTENTS (Continued)

CHAPTER 1

Introduction

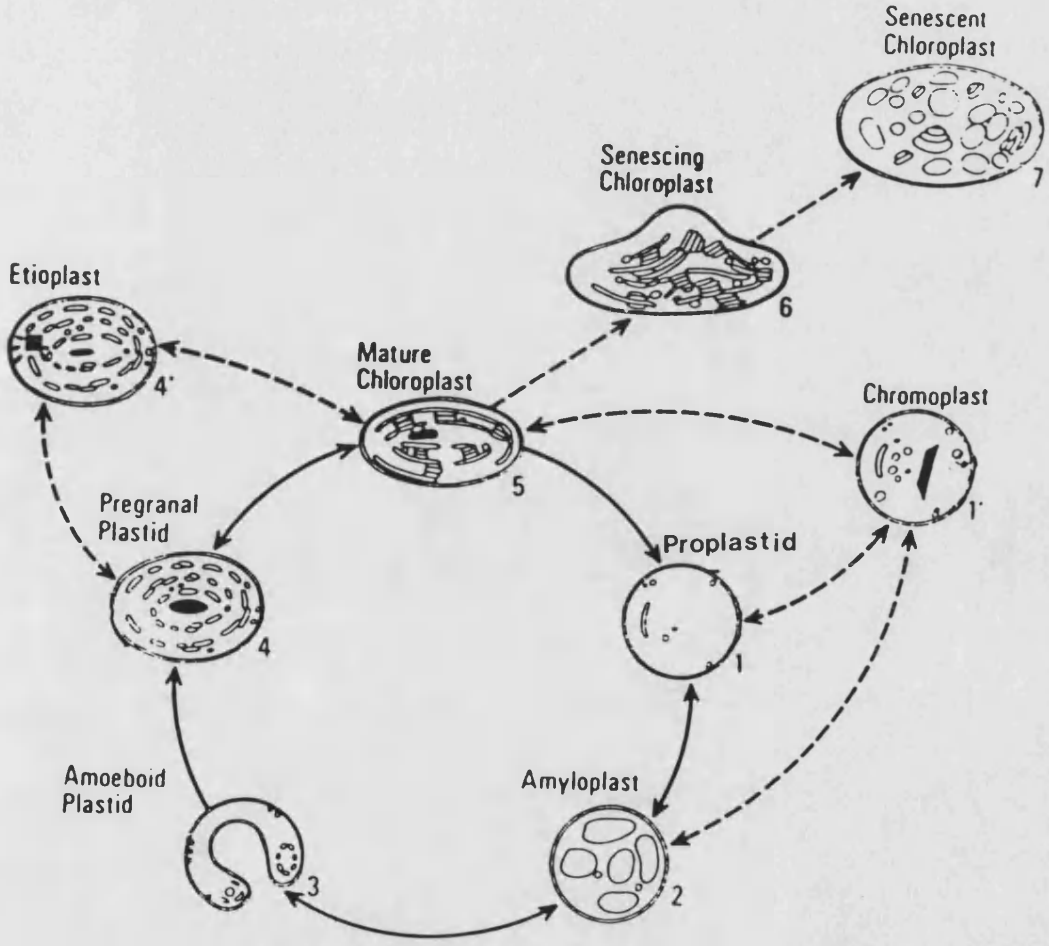
The nucleus and mitochondria are DNA-containing structures common to all eukaryotic cells, but in plant cells a third DNA-containing structure is found, the plastid. The plastid functions in such varied processes as photosynthesis, starch storage, carotenoid production, and lipid metabolism. The particular function of the plastid is dependent on the tissue in which it is located and also on environmental influences, such as light availability. With tissue development plastids change both in structure and function throughout the life-cycle of the plant, therefore it is obvious that some regulatory mechanism must be at work controlling these changes.

1.1 Development of Plastid Forms

In the late 1950's, Muhlethaler *et al.* (1959) suggested that plastids might arise from small submicroscopic vesicles that bud off from the nucleus. Since that time there has been no evidence to support *de novo* plastid formation, but extensive work involving light and electron microscopy have revealed plastids multiplying by binary fission. Examples of maternally inherited chloroplast traits in higher plants firmly established the theory of transmission of plastids through the female parent (Sager, 1972, 1977; Gillham, 1978). Thus, it is now generally accepted that new plastids arise from pre-existing plastids (Kirk and Tilney-Bassett, 1978; Possingham, 1980).

Plastids, as the name suggests, show plasticity in both structure and function. The precursor of the various plastid forms is the proplastid, which can be reformed from other plastid types throughout the life-cycle of the plant (Figure 1.1). Proplastids are found in the

Figure 1.1 Plastid developmental cycle (modified from Thomson and Whatley, 1980).



egg, pollen, zygote, ripe seed, and meristemic tissue of plants (Thomson and Whatley, 1980). They are small, undifferentiated organelles with a dense matrix containing many ribosomes and only a few stromal membranes bound by a double membrane. It is generally considered that the main function of proplastids is to act as precursors of the more differentiated types of plastids. Whether they have any other biochemical role is not known, although Kirk and Tilney-Bassett (1978) suggested that it is likely that they function in carbohydrate metabolism in the meristematic cells.

From proplastids, amyloplasts or chromoplasts may develop. Amyloplasts are mature plastids that function in starch synthesis and storage. They are found in differentiated cells of the root; and in storage tissues such as cotyledons, endosperms and tubers. They lack chlorophyll and appear to have a structureless stroma which is mainly taken up by starch. There may be one large, or several smaller, starch grains per amyloplast. In several plants there is evidence of a transitory association between the amyloplast and an encircling strand of endoplasmic reticulum (ER). It has been suggested that the amyloplast-ER complex may form a synthetic unit associated with carbohydrate metabolism and starch synthesis, with the ER functioning as a channel for transporting material to and from the plastid (Thomson and Whatley, 1980). It is not known whether the accumulation of starch in amyloplasts is the result of the absence of the necessary degradatory enzymes or if a state of imbalance exists between the rate of synthesis and the rate of degradation in storage tissues (Kirk and Tilney-Bassett, 1978; Jenner, 1982). Chromoplasts are carotenoid-containing plastids that are responsible for the red, orange, and yellow colours of fruits, flowers, and roots. Their precise function is unknown, but it is thought that they may act as an attractant for seed and pollen dispersal by birds, insects, *etc.*.

From amyloplasts, etioplasts may form in the leaves and cotyledons of plants grown in the dark. They contain most of the photosynthetic enzymes of chloroplasts (although in reduced amounts), many ribosomes, and 'prolamellar bodies'. The prolamellar bodies develop into thylakoid membranes with the subsequent development of the etioplast into a chloroplast.

Chloroplasts, the most widely researched of the plastids, develop from amyloplasts, chromoplasts, and etioplasts. Exposure to light is known to control the development of amyloplasts and etioplasts into chloroplasts. Extensive ultrastructural changes must occur for the development of chloroplasts, since their stromal matrix contains a complex system of sac-like membranes, known as thylakoids. The thylakoids are arranged in closely packed stacks, called grana, and lamellae, which connect the stacks. The thylakoids contain the components of the light reaction of photosynthesis, including the chlorophylls a and b found in higher plants. The function of the chloroplast, therefore, is to carry out photosynthesis, the process which enables the plant to utilise light energy to produce NADPH and ATP (Figure 1.2), which are then used to convert CO_2 to carbohydrate (Figure 1.3).

1.2 Plastid Autonomy and Origin

The ability of plastids to proliferate within cells, seemingly independent of any outside control, suggested that plastids are autonomous structures. In 1885 Schimper wrote, based on microscopic observation: "With respect to their reproduction and their chemistry they (the plastids) behave much more like independent organisms than like parts of the plasmic body; they show no relation to the cytoplasm or the nucleus and despite various metamorphoses they retain their characteristics throughout the plant kingdom" (translation by Diers, 1970).

Figure 1.2 Schematic representation of photosynthetic electron flow (—) and proton transport (---) in thylakoid membranes upon activation by light (from Anderson and Andersson, 1982).

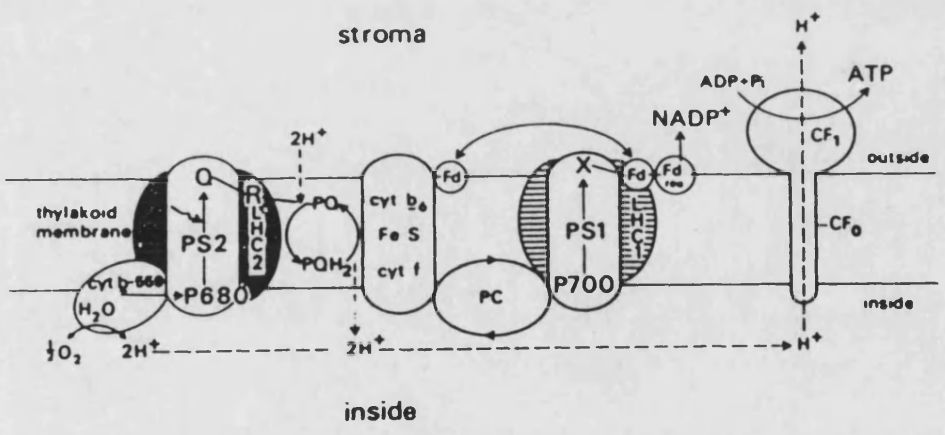
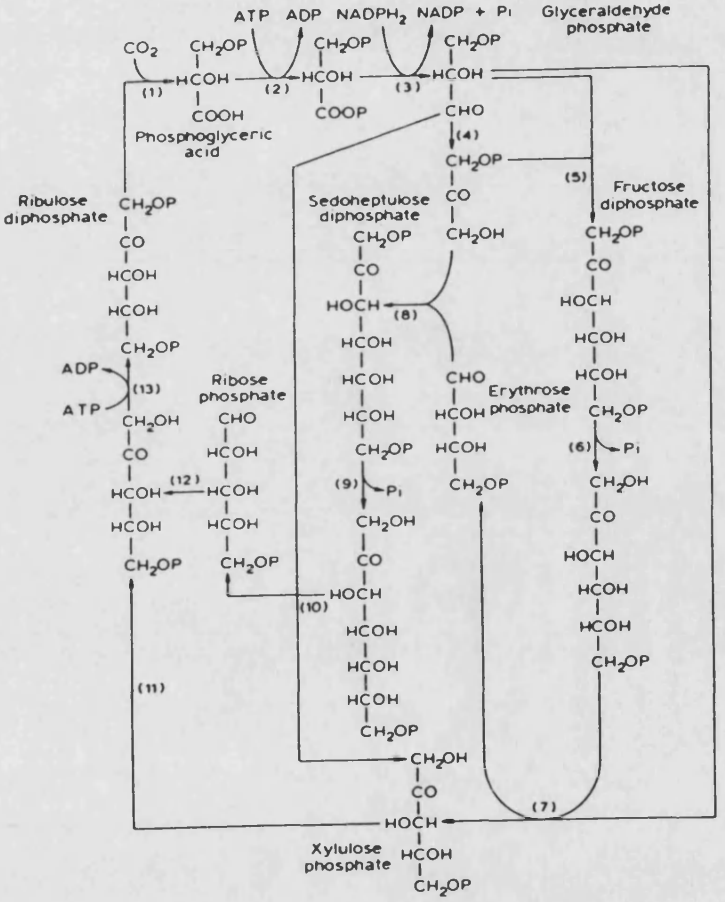


Figure 1.3 The pathway in the photosynthetic CO_2 fixation cycle. P - phosphate group, Pi - inorganic phosphate. The enzymes involves are (1) RuBPcarboxylase, (2) 3-phosphoglyceric acid kinase, (3) glyceraldehyde-3-phosphate dehydrogenase, (4) triose-phosphate isomerase, (5) fructose-diphosphate aldolase, (6) fructose 1,6-di-phosphatase, (7) transketolase, (8) aldolase, (9) sedoheptulose-1,7-diphosphatase, (10) transketolase, (11) xylulose-5-phosphate epimerase, (12) ribose-5-phosphate isomerase, (13) ribulose-5-phosphate kinase.



Now, plastids are known not to be entirely independent of the rest of the cell and are instead referred to as being 'partially' autonomous structures. For a structure to be considered autonomous it must contain four components: (1) DNA to code for its entirety, (2) DNA polymerase to replicate the DNA, (3) RNA polymerase to transcribe the DNA, and (4) protein-synthesising machinery to translate the mRNAs into all the necessary proteins. Chloroplasts contain all four components, but their DNA does not code for all the chloroplast proteins, nor does their protein-synthesising machinery make all the chloroplast proteins (for reviews see: Tewari, 1971; Ellis, 1976; Gillham *et al.*, 1978; Chua and Schmidt, 1979; Bradbeer, 1981; Ellis, 1981; and Ledoigt and Freyssinet, 1982).

The similarities plastids have to a population of symbiotic unicellular micro-organisms multiplying within the cells of a host led to the theory of endosymbiosis to explain the compartmentalisation of DNA in the cells of higher plants. However, evidence for the possible evolutionary origin of plastids is limited to organisms living to-day. The cyanelles of the alga *Cyanophora* are thought to be modern intermediates representing an evolutionary step between free-living cyanobacteria (prokaryotic blue-green algae) and chloroplasts (Aitken and Stanier, 1979; Whatley *et al.*, 1979; Ellis, 1983; Ko *et al.*, 1985). Some of the similarties between the cyanobacteria and the cyanelles include the presence of unstacked thylakoid membranes, and the photosynethetic pigments, chlorophyll a and accessory phycobilins. The genomes of the cyanelle and cyanobacteria are also similar in that both code for the large and small subunits of RuBPcarboxylase, rbcL and rbcS, and for the β chain of phycocyanin (Heinhorst and Shively 1983; Lemaux and Grossman, 1984). Despite these similarities between

cyanelles and cyanobacteria, there appear to be many similarities between the plastome of higher plant chloroplasts and the genome of the cyanelle. The cyanelle genome has been analysed for homology to specific gene probes known to be encoded on the chloroplast plastome (rbcL, the α, β and ε subunits (atpA, atpB, atpE) of the CF1 of ATP-synthase, subunit III (atpH) of CFo of ATP-synthase, the 32-kilodaltons (kd) thylakoid membrane protein (psbA), cytochrome f (petA), and the ribosomal subunit genes for the 16S and 23S rRNA). In this study, Ko *et al.* (1985) found that the cyanelle appeared to retain all these gene sequences. Ko *et al.* (1985) also demonstrated that every fragment of the broad bean ct DNA library shows homology to regions of the cyanelle genome.

The discovery of the prokaryotic alga, *Prochloron,* which has both the chloroplast chlorophylls, a and b, (Lewin and Withers, 1975; Thorne *et al.*, 1977; Withers *et al.*, 1978) stacked thylakoids (Thinh, 1978; Giddings *et al.*, 1980; Cox and Dwarte, 1981), lack of phycobiliproteins and phycobilisomes provided a living example of a possible chloroplast precursor. However, no prochlorophyte (the phylum to which *Prochloron* belongs) has been found to occur as an endosymbiont, nor has an intermediary stage of endosymbiosis been found (Whatley *et al.*, 1979).

The many similarities between chloroplasts and free-living oxygenic-photosynthetic prokaryotes: DNA size and GC content, RNA size, gene sequences of cytochromes and ferredoxins, thylakoid arrangement and chlorophyll content (Whatley *et al.*, 1979; Gray and Doolittle, 1982); leave little doubt that chloroplasts arose from a prokaryotic ancestor. However, the evidence could allow for the entire eukaryotic cell having originated from a free-living oxygenic-photosynthetic prokaryote which subsequently underwent evolutionary divergence and compartmentalisation

(Gray and Doolittle, 1982). This theory would help to explain the presence of structural genes concerned with the synthesis of chloroplast proteins in the nucleus. Raff and Mahler (1972) suggest that the organellar DNAs might have been plasmids dervied from the main chromosome. Uzzell and Spolsky (1974) have proposed that initially vesicles carried all the DNA and eventually through specialisation of function with selective loss of other activities gave rise to plastids, mitochondria and the nucleus (Kirk and Tilney-Bassett, 1978). The question of whether the plastids originated from encapsulation of photosynthetic machinery within an ancestral oxygenic-photosynthetic prokaryotic cell (direct filiation or autogenous origin hypothesis) or from endosymbionts harboured in an originally non-photosynthetic host (endosymbiont or xenogenous origin hypothesis) is still unanswered.

1.3 Plastid DNA

With the discovery in the late 1950's that genetic information is carried in the form of DNA (or RNA in certain viruses) in all living organisms, a search for DNA in plastids began. In the 1960's, electron microscopy, base composition, and buoyant density determinations all helped to establish plastid DNA as unique from nuclear DNA.

The plastid DNA, or plastome, in higher plants has a BC content of 37.8 ± 1% and a buoyant density in CsCl of 1.697 ± 0.001 g cm^{-3}. The plastome exists *in vivo* as a circular molecule with a circumference of 40-46 μm or 120-190 kbp. Electron microscopy has shown that these DNA circles are usually grouped into nucleoids with the average nucleoid containing about four circles (Kirk and Tilney-Bassett, 1978).

Plastid DNA was found to resemble prokaryotic DNA in that it is not associated with histones (Ellis, 1976) and it contains no methylated

cytosines (except in the chloroplast DNA of Chlamydomonas, Sager *et al.*, 1984), whereas nuclear DNA contains a small amount of 5-methylcytosine. Recent evidence shows that nuclear DNA contains sequences of plastid DNA, possibly enough to duplicate the information in the entire plastid DNA (Scott and Timmis, 1984b), but these genes may be in an interrupted, highly methylated and perhaps inactive form. There is also evidence of the presence of pt DNA sequences in mitochondrial DNA (Stern and Lonsdale, 1982; Stern and Palmer, 1982/1983 ; Stern and Palmer, 1984). The strongest cross-homologies observed have been with sequences unlikely to function in the mitochondrion: the large subunit of RuBPcarboxylase and the plastid rRNA genes. Stern and Palmer (1982/1983a) feel the presence of pt DNA sequences in plant mitochondrial DNAs demonstrates the dynamic interaction between the chloroplast and mitochondrion; similar to that found between the mitochondrion and the nucleus, where inter-organellar DNA transfer has been documented (van den Boogaart *et al.*, 1982; Wright and Cummings, 1983; Gellissen *et al.*, 1983; Farrelly and Butow, 1983; Jacobs *et al.*, 1983). However, exchange of DNA sequences between organelles may not involve direct physical contact. The DNA of lysed or broken plastids released into the cytoplasm may be taken up by the mitochondria or nucleus by transformation (Stern and Palmer, 1982/1983); or as mentioned previously, there is the possibility of a common origin for the plastids, nucleus and mitochondria (direct filiation theory) which would explain the presence of similar gene sequences in the organelles.

1.3.1 Structure and complexity

From restriction enzyme mapping and hybridisation tests it has been determined that the plastome is divided into four regions in many plants, two inverted repeat regions separated by a small and a

large single-copy region (Bedbrook and Kolodner, 1979). The inverted repeat (Figure 6.2), consisting of approximately 20-25 kbp, has been found in a majority of angiosperms studied as well as in *Spirodela* (van Ee *et al.*, 1980) and the cyanelles of *Cyanophora paradoxa* (Bohnert and Loffelhardt, 1982). The only documented loss of this inverted structure has occurred within one section of the family *Leguminosae*; pea, chick pea (Chu and Tewari, 1982), broad bean (Koller and Delius, 1980) and alfalfa (Palmer and Thomson, 1982/1983). Thus, there are two types of plastome in the family *Leguminosae*, one that contains the inverted repeat (*e.g.*, soy bean Chu and Tewari, 1982; and mung bean Palmer and Thompson, 1981) and one that does not (*e.g.*, pea, broad bean and chick pea). The inverted repeats contain the genes for ribosomal and tRNAs and seems to be a highly conserved arrangement. There appears to exist a strong correlation between the presence of the inverted repeat and stability within the chloroplast genome (Palmer and Thompson, 1982/1983). Numerous rearrangements have occurred within those species which have lost the inverted repeat and those that have retained it show few major sequence rearrangements (Palmer and Thompson, 1982/1983). Alfalfa pt DNA is considered a 'missing link' in that it has lost one entire segment of the inverted repeat but is otherwise colinear in sequence arrangement relative to those legume pt DNAs that retain the repeat (Palmer and Thompson, 1982/1983).

Tassopulu and Kung (1984) found most alterations in the *Nicotiana* plastome occur in the large single-copy region, particularly near the right-hand border of the inverted repeat. This region has been designated the 'hot spot'. From studies on various *Nicotiana* species one, *N. acuminata*, contains an extra fragment in the 'hot spot' which extends the symmetry of the inverted repeat (Shen *et al.*, 1982). This has led to the proposal that present plastomes may have evolved from a

larger plastome which consisted of two identical copies joined head-to-head and tail-to-tail fashion (Tassopulu and Kung, 1984).

1.3.2 Changes in plastome level during development

The percentage of plastid DNA in total cell DNA (*i.e.*, nuclear, mitochondrial and plastid) changes during plant development and varies from tissue to tissue. For example, plastome levels in leaves of tobacco (4%), pea (12%), spinach (21%), and potato (7.6%) (Siegel, 1974; Lamppa and Bendich, 1979; Scott and Possingham, 1983; Scott *et al.*, 1984a) have been found to be higher than in roots (1.0%, 0.4%, 2.1%, 1.0%, respectively for tobacco, pea, spinach and potato). Lamppa and Bendich (1979) also found a 13% increase in plastome level in fully greened pea leaves when compared to embryos and etiolated tissue. Scott *et al* (1984a) found higher levels of pt DNA in potato leaf tissue (7.6%) compared to petioles (3.4%) and stem (3.0%).

There is a considerable body of information on the influence nuclear ploidy has on plastid number (Scott and Possingham, 1982a). This relationship has been extensively reviewed by Butterfass (1979), who came to the conclusion that the number of chloroplasts per cell is controlled and limited by the amount of nuclear DNA. This conclusion was found to apply mainly to meristemic cells, where the replication of nuclear DNA led to the replication of plastids, immediately, or some time later, but before the next replication of nuclear DNA occurred. For those cells which were no longer meristematic, there was a quantitative deviation from the strict correlation between the amount of nuclear DNA and the number of plastids. The reason for the break in the correlation is unknown; Butterfass (1979) attributes it to inefficiency in control due to the change in function of the cell.

Unfortunately, there is not enough experimental evidence to include plastid DNA levels also in these correlations. Lamppa and Bendich (1979) found dark-grown pea epicotyls increase their nuclear ploidy levels by at least a factor of 4, and the synthesis of pt DNA appears to keep pace with nuclear DNA endoreduplication in dark-grown shoots, but not exceed it. In contrast, low levels of pt DNA were found in roots (see above), which Lamppa and Bendich (1979) explained by nuclear DNA increases unaccompanied by pt DNA replication. Scott *et al.* (1984a) suggested a possible link between nuclear and plastid DNA replication in potato tissue. Potato leaf tissue (4C) was found to have 22 plastome copies per plastid, while tuber tissue (average 14C) was found to have 195 plastome copies per cell.

1.4 Plastid Ribosomal RNA

Lyttleton (1962) was the first to show that plant cells contain two classes of ribosome, 70S ribosomes in the chloroplast and 80S ribosomes in the cytoplasma. The chloroplast ribosome consists of two subunits, a 50S subunit and a 30S subunit. The large subunit contains a 23S rRNA with a molecular weight of 1.1×10^6, and the small subunit contains a 16S rRNA with a molecular weight of 0.56×10^6. The chloroplast ribosomes and their RNA components resemble those found in prokaryotic cells. The similarities were thought to be in size only (Ellis, 1976); however recent studies show that homologies do exist between chloroplast and *E. coli* rRNAs (Schwarz and Kössel, 1980). Also, the genes for chloroplast ribosomal proteins have been found to be homologous to *E. coli* genes coding for ribosomal proteins (Sugita and Sugiura, 1983; Watson and Surzyaki, 1983). Dorne *et al.* (1984) compared the ribosomal proteins from *E. coli* and from chloroplasts of spinach using electro-

phoretic migration and immunochemical cross-reaction between blotted *E. coli* ribosomal proteins and chloroplast subunit antisera. Their findings suggest the presence of at least one homologous ribosomal protein in the 50S subunit of *E. coli* and spinach chloroplasts; and one, possibly three, 30S *E. coli* ribosomal proteins immunologically cross-reacted with a chloroplast 30S antiserum. Very recently, Shinozaki *et al.* (1986) found a tobacco ct DNA nucleotide sequence encoding a protein homologous to an *E. coli* ribosomal protein.

Chloroplast ribosomes constitute about 17% of the total (cytoplasmic plus chloroplast) ribosomes in wheat leaves (Jones *et al.*, 1973), about 30% in pea (Boardman, 1966), and about 60% in young tobacco leaves (Bourque *et al.*, 1973). Most of the RNA in plastids is rRNA but lesser amounts of low molecular weight 4.5S, 5.0S, tRNAs and mRNAs are also found.

Becker *et al.* (1978) found that chloroplast rRNA molecules in cucumber seedlings increased from zero at day 1 (first day from planting of the cold-imbibed seeds) to 26 million per cell by day 7. Dean and Leach (1982) found increases in 70S ribosomes during the development of the first leaf of wheat to be 19×10^5/h. Using quantitative electron microscopy, the authors also found a 3-fold increase in 70S ribosomes per plastid during the development of young corn leaves.

1.5 Protein Synthesis in Plastids

If the approximately 40 μm circle of plastid DNA consists of unique base sequences, it is sufficient in principle to code for about 125 proteins each of molecular weight 50,000. So far 190 resolvable chloroplast polypeptides have been found when stromal and thylakoid preparations have been analysed. However, the total molecular weight of chloroplast

polypeptides detected exceeds the total potential coding capacity of the chloroplast plastome. Thus, it can be concluded that not all the chloroplast polypeptides are encoded on the plastome (Ellis, 1981).

1.5.1 *In vivo* studies

In vivo antibiotic inhibition experiments were used to determine the sites of synthesis of chloroplast proteins (for reviews of inhibition studies see Ellis, 1981; Kirk and Tilney-Bassett, 1978). The most commonly used inhibitors are D-chloramphenicol, used to inhibit organellar protein synthesis (*i.e.*, chloroplast) and cycloheximide, which inhibits protein synthesis of cytoplasmic ribosomes. This approach has limitations in that these antibiotics also disrupt other processes in addition to protein synthesis (Ellis, 1981). The results from carefully controlled inhibitor experiments suggest that most of the chloroplast proteins so far examined are synthesised on cytoplasmic ribosomes (Ellis, 1981). The synthesis of enzymes of the Calvin cycle: ferredoxin, RNA polymerase, aminoacyl-tRNA synthetases, and the majority of the thylakoid and ribosomal polypeptides (but not RuBPcarboxylase) are not affected when protein synthesis in the chloroplast is inhibited (Ellis, 1981). Besides RuBPcarboxylase, the other proteins dependent on chloroplast ribosomal activity are elongation factors, the chloroplast coupling factor, and several chloroplast ribosomal and thylakoid polypeptides, including cytochromes (Chua and Gillham, 1977; Hoober, 1976).

1.5.2 *In vitro* studies

Studies of *in vitro* protein synthesis in isolated chloroplasts using the radiolabel ^{35}S-methionine showed that the major soluble protein product synthesised is the large subunit of RuBPcarboxylase

(Blair and Ellis, 1973; Bottomley *et al.*, 1974). Interestingly, *in vitro* protein synthesis studies also showed that the small subunit of RuBPcarboxylase is synthesised on cytoplasmic polysomes (Gray and Kecwick, 1974). It has also been found that other polypeptides are synthesised in the chloroplast using *in vitro* analysis. The second major protein product is an insoluble polypeptide known as the 32 kd protein, or peak D (Ellis, 1981). In addition, about 90 minor products have been resolved using two-dimensional separation of ^{35}S-methionine labelled protein products (Ellis, 1981). The identity of most of these is unknown. Table 1.1 summarises the identified products of *in vitro* chloroplast protein synthesis.

1.5.3 Studies on plants with ribosome-deficient plastids

There seems to be excellent agreement between the results of *in vivo* and *in vitro* work for determination of the site of protein synthesis in certain plant species (Ellis, 1981). An alternative method to study the site of chloroplast protein synthesis used plants deficient in plastid ribosomes, either genetic mutants or heat-treated plants. The heat-treated plants lacking plastid ribosomes confirmed the results obtained from inhibitor and *in vitro* approaches. The plastome mutants also showed that cytoplasmic ribosomes are needed to synthesise the membranes of the plastid envelope and to make a rudimentary thylakoid membrane, although these membranes were not necessarily normal or functional (Ellis, 1981; Kirk and Tilney-Bassett, 1978).

1.5.4 Transport of cytoplasmic proteins into the chloroplast

The proteins made in the cytoplasm but needed for various functions inside the chloroplast must cross the double membrane of the chloroplast.

Table 1.1 Identified products of *in vitro* chloroplast protein synthesis

Polypeptide
Large subunit of RuBP carboxylase
Three or four subunits of the chloroplast coupling factor (CF_1)
Elongation factors T and G of chloroplast protein synthesis
Cytochrome f
Cytochrome b559
Dicyclohexylcarbodi-imide-binding protein
Apoprotein of chlorophyll-protein complex I
Thylakoid membrane protein (32-kd)

(fromEllis, 1981)

Both the small subunit of RuBPcarboxylase and the chlorophyll a/b binding protein enter the chloroplast and the small subunit of RuBPcarboxylase is assembled into functional RuBPcarboxylase, while the chlorophyll a/b binding protein is integrated into the thylakoids and assembled into a complex with chlorophyll (Chua and Schmidt, 1978a; Chua and Schmidt, 1978b; Chua and Schmidt, 1979; Dobberstein *et al.*, 1977; Ellis *et al.*, 1980; Highfield and Ellis 1978; Schmidt *et al.*, 1980; Smith and Ellis, 1979). It has been found that the mechanism of transport is post-translational and involves a specific interaction between the finished polypeptide precursor and the chloroplast envelope (Blobel, 1980; Dobberstein *et al.*, 1977; Highfield and Ellis, 1978). The extra amino acid sequences in the precursor probably functions in the recognition interaction with the chloroplast envelope. Ellis (1981) predicted that the extra precursor sequence is the same for all polypeptides (within the same plant) destined to enter the chloroplast. Recently it has been demonstrated that although the precursor sequences are rich in basic amino acids, they show little homology (Hoober, 1984).

From *in vitro* protein synthesis studies, *in vivo* inhibition studies, and investigations on plants deficient in plastid ribosomes it appears that most chloroplast proteins are formed in the cytoplasm, some in the chloroplast, and other require the co-ordinated activities of both cytoplasmic and chloroplast ribosomes (*e.g.*, RuBPcarboxylase).

1.6 Use of Potato Tuber Tissue to Study the Transformation of Amyloplasts into Functional Chloroplasts

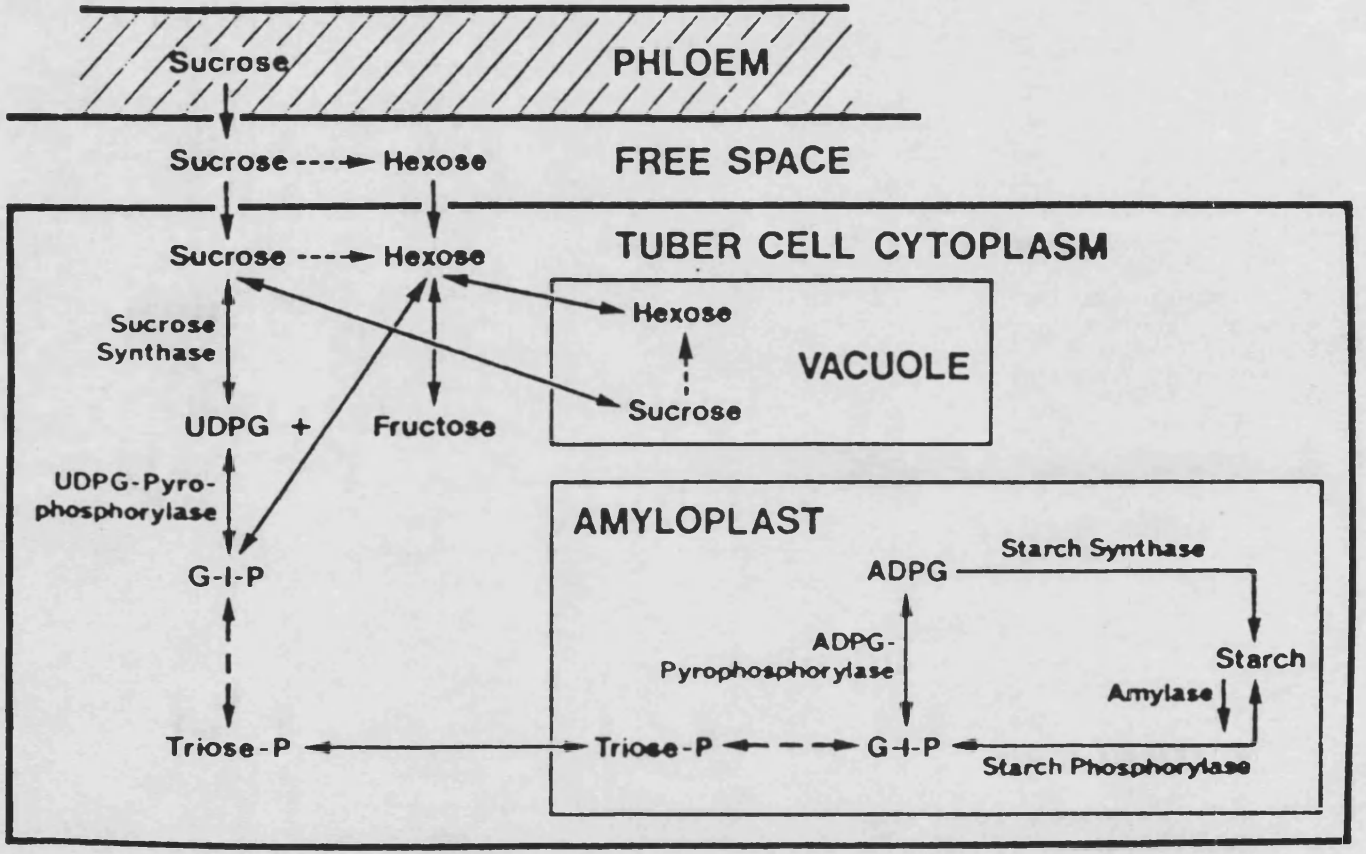
1.6.1 The potato tuber

The potato tuber is a shortened stem which has differentiated into a storage organ. The first indication of tuber formation is a slight swelling of the ultimate and penultimate elongation internodes behind the terminal bud of the stolon (Slater, 1963). Initiation of tuberisation, results in a loss of polarity with respect to the

plane of cell division causing radial cell expansion and division instead of the typical longitudinal growth (Slater, 1963; Lovell and Booth, 1967; and Park, 1984). There are two theories for the initiation of tuber formation, which are not necessarily exclusive of one another. One suggests that tuber initiation is due to the build-up of high levels of soluble carbohydrate at the stolon tip under conditions which depress the growth of the shoot (*i.e.*, short day length and low temperatures). The other proposes 'hormonal' control of tuberisation, with a 'hormone-like' substance coming from the leaves during short days (Lovell and Booth, 1967; and Park, 1984). The exact molecular mechanism controlling tuberisation is unknown, but an interplay between these two mechanisms seems likely.

The onset of tuberisation is accompanied by a rise in the content of starch per cell. Unlike other storage organs, such as cereal grains, where the movement or diffusion of sucrose from the phloem to the storage cell is restricted by the situation of the phloem in relation to the storage cells (Jenner, 1974); the potato tuber contains many phloem strands (Artschwager, 1924) and there is presumably a close connection between transporting and storage tissues. Photosynthate from the phloem must cross the apoplast (free space) before it enters into the storage cell (Figure 1.4). Once inside, it is generally agreed that starch synthesis is catalysed by the enzymes ADPG-pyrophosphorylase and starch synthase, with starch phosphorylase being primarily involved in starch degradation (Mares and Marschner, 1980). The transport of soluble sugar across the amyloplast membrane is assumed to be mediated by the phosphate translocator previously demonstrated in chloroplasts by Heldt (1976). Unfortunately, little is known about the transport of sugar into

Figure 1.4 Schematic outline of the movement of sugar from the phloem sieve elements, through the apoplast (free space) to the storage parenchyma (tuber cell) and a probable pathway for its subsequent conversion to starch. (From Mares and Marschner, 1980).



the amyloplast due to the difficulty in isolating intact amyloplasts. (The large starch granules tend to disrupt the amyloplast structure during isolation procedures.) As the tuber continues to grow, starch accumulates at a faster rate (for an excellent review of starch storage see Jenner 1982); eventually growth and starch accumulation declines. The mechanism controlling the cessation of growth is unknown. Net breakdown of starch associated with sprouting does not follow immediately, but occurs after a considerable amount of time.

1.6.2 The amyloplasts of potato tubers

One function of the cells of the potato tuber is to accumulate and store carbohydrate in the form of starch granules in amyloplasts. Kirk and Tilney-Bassett (1978) suggest that amyloplast formation in any tissue requires a plentiful supply of sugars and may also require a considerable increase in the amount of the various enzymes required for starch synthesis. From studies with maize and barley mutants several genes have been found which control starch synthesis. These starch synthesis genes all show Mendelian inheritance, and thus appear to be located in the nuclear genome (Kirk and Tilney-Bassett, 1978). Evidence from studies with plastid mutants which lack plastid ribosomes, also suggest that nuclear genes code for the enzymes involved in starch accumulation and synthesis (Hagemann and Borner, 1978). It may, therefore, be thought that plastome levels should be very low in amyloplasts. However, Scott *et al.* (1984a) found unexpectedly high levels of the plastome in amyloplasts of potato tubers. They found 5.2% plastid DNA in total DNA extracts (nuclear, mitochondrial and plastid DNAs) of potato tubers compared to 7.6% found in potato leaf tissue, while root tissue had only 1.0% plastid DNA in the total DNA extract. The plastome copy number

per plastid in amyloplasts of the tuber was approximately ten times the copy number found in potato leaf chloroplasts, with about 40 plastids per cell in tuber tissue and 135 in leaf tissue. The reason for this high level of plastid DNA in potato tuber amyloplasts is not fully understood. Scott *et al.* (1984a) suggested that there may be some, as yet, undetermined function in amyloplasts requiring a large number of available cistrons for transcription; this has yet to be resolved. Alternatively, the high pt DNA levels in tuber tissue could be the consequence of the high nuclear ploidy levels found in many storage tissues, including the potato (Scott *et al.*, 1984a). Lobov and Bondar (1977) reported that rRNA components are not present in potato tuber amyloplasts. The only RNA they found in amyloplasts was a low-polymeric fraction that resembled tRNA, which was unable to accept labelled amino acids. The authors therefore concluded that potato tuber amyloplasts do not possess a protein synthesising system.

1.6.3 Light-induced transformation of amyloplasts into functional chloroplasts

Upon exposure to light, the outer tissues of potatoes tubers will turn green within several days, this greening indicates the formation of chlorophyll and the transformation of amyloplasts to chloroplasts. Thus, the potato tuber provides an excellent tissue with which to study the development of amyloplasts to chloroplasts. Upon transformation, invaginations of the inner envelope of the amyloplast begin and eventually form the thylakoids, grana differentiate and ribosomes appear in the stroma (Zhu *et al.*, 1984). From studies on potato tuber discs Zhu *et al.* (1984) have provided biochemical evidence to confirm the transformation of amyloplasts into chloroplasts.

They have shown that before illumination potato tubers contain no chlorophyll or protochlorophyll, but after illumination chlorophyll appears. The chlorophyll content was found to be only about one hundredth of the chlorophyll content found in normal leaves, which is most likely due to there being fewer chloroplasts per cell. RuBPcarboxylase and other proteins (unidentified) were found to be synthesised *de novo* during greening. The *de novo* synthsis of RuBP-carboxylase was clearly demonstrated by ultracentrifugal sedimentation and specific immunoprecipitation. These results were further confirmed by analysis of the protein products of light-driven protein synthesis in the isolated chloroplast of illuminated potato tissue. Furthermore, the photosynthetic function of electron transport and O_2 evolution were shown by high Hill reaction activities in isolated chloroplasts of potato tuber. This evidence verified that the light reaction of photosynthesis and ATP formation were functioning as in normal chloroplasts and also that RuBPcarboxylase was present to initiate CO_2 assimilation.

1.7 Aims of Investigations Undertaken

Most data available on plastid transformation concerns the development of etioplasts to chloroplasts. Very little research has been done on amyloplast to chloroplast transformations. This thesis describes investigations into some molecular aspects of the light-induced transformation of amyloplasts to functional chloroplasts in potato tuber tissue.

There were two initial aims in this work. The first was to determine the level of pt DNA in white and green tuber tissue, in comparison to that found in leaf tissue. The major function of amyloplasts in white tuber tissue is that of starch synthesis and

storage. However, data show that the enzymes involved in starch synthesis are nuclear-encoded, suggesting that only very low levels of pt DNA are required in white tuber tissue. With 'greening' of the tuber the many chloroplast-encoded enzymes involved in photosynthesis are required. The determination of plastome level in white and green tuber tissues would therefore provide information on whether the plastome is amplified during its change of function in the greening process and whether low levels of pt DNA are indeed found in white tuber tissue.

The second aim was to determine the activity and coding capacity of the plastome in white and green tuber tissues. Polyacrylamide gel electrophoresis, reassociation rate kinetics, hybridisation analysis and hybrid-release translation techniques were used to determine the level of RNA transcripts and their products found in green and white tuber tissues. It was hoped that these studies would provide a clearer understanding, not only of the transformation mechanism, but also of the possible function of amyloplast DNA.

CHAPTER 2

Materials

2.1 Plant Material

The plant species used in all of the following experiments was potato (*Solanum tuberosum L.*, var. King Edward). The tubers were purchased locally and stored in the dark until use, usually within a week of purchase. Green potato tubers resulted from exposing surface-cleaned white potato tubers to varying periods of illumination in an illuminated incubator (Gallenkamp Cooled Incubator, model INF-781-T, fitted with cool white fluorescent light tubes) at 25° C with 14 h daily illumination at 26 $\mu E\ m^{-2}\ s^{-1}$. (These green potato tubers are differentiated by their illumination period; *i.e.*, 4 days, 8 days, or 12 days, in future references.) Leaf material was obtained from plants grown from tubers, potted close to the soil surface in Levington potting compost, in a greenhouse supplemented with sodium lamps (400 W) at 14-25° C, depending on the time of year. Well established plants from which leaf material could be taken took approximately 6-8 weeks to grow.

2.2 Chemicals and Solvents

Bactoagar, bactotryptone, and yeast extract were purchased from Difco Laboratories, Detroit, MI, U.S.A. Nutrient broth was supply by Oxoid Ltd., Basingstoke, U.K. Sodium dodecylsarcosinate (*N*-laurylsarcosine, sodium salt), cesium chloride, and ethidium bromide (2,7-diamino-10-ethyl-9-phenyl-phenanthridium bromide), agarose (type II, medium), dithiothreitol (DDT), ampicillin, tetracycline, chloramphenicol, blue dextran, methylene blue, diphenylamine, salmon testes sperm DNA, ficoll,

polyvinylpyrrolidone (PVP), bovine serum albumin (BSA), transfer RNA (tRNA), Coomassie brilliant blue R250, dalton marker VI (containing: lysozyme, β-lactogloblin, trypsinogen, pepsin, egg albumin, and bovine albumin), thiamine and TEMED (*N,N,N',N'*-tetramethyl-ethylenediamine) were purchased from Sigma Chemical Co. (U.K.), Poole. Formamide and 2-mercaptoethanol were from Aldrich Chemical Co. Inc., Milwaukee, WI, U.S.A. The radiolabelled nucleotides, ^{3}H-TTP ((methyl-^{3}H) thymidine 5'-triphosphate, ammonium salt), ^{35}S-CTP (deoxycytidine 5'-(α-^{35}S thio) triphosphate, triethyl-ammonium salt), and the radiolabelled amino acid ^{35}S-methionine were supplied by Amersham International Plc, Amersham, U.K.; as was the amino acid depleted rabbit reticulocyte lysate and the unlabelled amino acids minus methionine. Sephadex G-50 (fine) was from Pharmacia Fine Chemicals AB, Uppsala, Sweden. X-gal (5-bromo-4-chloro-3-inddyl-β-*D*-galactoside) and IPTG (isopropythio-β-*D*-galactoside) were from Bethesda Research Laboratories (U.K.), Cambridge. All other chemicals were from either Fisons Scientific Apparatus, Loughborough, U.K., or BDH Chemicals Ltd., Poole, U.K. All solvents used in the experimental procedures were from either May and Baker Ltd., Dagenham, U.K., or Fisons Scientific Apparatus.

2.3 Enzymes

Macerozyme R-10 was from Yakult Pharmaceutical Industry Co. Ltd., Japan. DNAse I, RNAse-A, protease, lysozyme, and S1 nuclease (from *Aspergillus oryzae*, type III) were from Sigma Chemical Co. (U.K.), Poole. The restriction endonucleases Bam HI and Pst I were from Bethesda Research Laboratories (U.K.) Ltd., Cambridge; Sal I was from P.L. Biochemicals, Inc., Milwaukee, WI, U.S.A.; Eco RI was from Sigma Chemical Co.; and Hind III was from Boehringer Mannheim Gmbh, West

Germany. The Nick Translation Kit was from Bethesda Research Laboratories (U.K.) Ltd., Cambridge (Table2.1).

2.4 Reagents

The Feulgen reagent was a gift from Keith Edwards, Corporate Bioscience Laboratories, Imperial Chemical Industries Plc, Runcorn, U.K.

2.5 Bacterial, Bacteriophage, and Plasmid Strains

The *E. coli* strain WL87 and the bacteriophage λ (wild type) were gifts from W. Brammar, University of Leicester, U.K. The *p*BR322 vectors containing the cloned 9.6 kbp wheat chloroplast DNA fragment (B2) and the 8.4 kbp wheat chloroplast DNA fragment (P6) were gifts from Catherine Bowman and Tristan Dyer, Plant Breeding Institute, Cambridge, U.K. The *E. coli* K12 strain HB101 was obtained from the Microbiology Department, University of Bath. The wheat chloroplast α and β subunits of ATP synthase and cytochrome f genes cloned into the filamentous coliphage M13 DNA were gifts from Chris Howe, University of Cambridge, Cambridge, U.K. The *E. coli* K12 strain JM107 was a gift from Keith Edwards, Corporate Bioscience Laboratories, Imperial Chemical Industries Plc, Runcorn, U.K.

2.6 Glassware and Plasticware

All glassware and plasticware which was to come into contact with DNA or RNA samples was first silicon-coated to prevent DNA or RNA adhesion to the surface and then sterilised by autoclaving. The items to be siliconised were soaked or rinsed in dimethyldichlorosilane solution (about 2% in 1,1,1-trichloroethane) and allowed to dry. These items were then rinsed thoroughly in water and autoclaved at 104 kPa for 20 min. Dimethyldichlorosilane is toxic and highly volatile, and therefore should be used only in a chemical hood.

Table 2.1

Components of the BRL Nick-translation reagent kit

Solution A

0.2 mM dATP, dGTP, dTTP (for use with ^{35}S-CTP)

0.2 mM dATP, dCTP, dGTP (for use with ^{3}H-TTP)

0.50 M-Tris-HCl (pH 7.8)

0.05 M-$MgCl_2$

0.10 M-2-mercaptoethanol

100 µg ml^{-1} nuclease-free BSA

Solution C

0.4 units $µl^{-1}$ DNA polymerase I

40 µg ml^{-1} DNAse I

0.05 M-Tris-HCl (pH 7.5)

0.005 M-Mg-acetate

0.001 M-2-mercaptoethanol

0.1 mM PMSF

50% glycerol

100 µg ml^{-1} nuclease free BSA

Solution D (stop buffer)

0.3 M Na_2EDTA (pH 8.0)

CHAPTER 3

Methods

3.1 Extraction and Analysis of Chlorophyll Content in Potato Tubers Exposed to Light

Potato tubers exposed to 0, 4, 7, 10 and 17 days of illumination were assayed for total chlorophyll content according to the methods of Anstis and Northcote (1973) and Arnon (1949).

After the illumination period, tubers were scrubbed clean and the outer skin was removed. Two grams of the periderm and the outer layer of parenchyma tissue were removed and homogenized with a mortar and pestle in 80% (v/v) acetone. To aid in the extraction of chlorophyll, sterile sand and some anhydrous sodium carbonate were also added. The mixture was then centrifuged for 5 min at 400 g in a bench centrifuge (MSE Scientific Instruments). The pellet was discarded and the absorbance of the chlorophyll/acetone solution was measured against a blank of 80% acetone at 645, 663 and 700 nm on a spectrophotometer (Pye Unicam, SP6-540, uv/VIS spectrophotometer).

3.2 Determination of Nuclear DNA Ploidy in Individual Nuclei from Various Potato Tissues

The procedure for the preparation and staining of tissues with Feulgen reagent was adapted from McLeish and Sunderland (1961). Treatment of tissues with Macerozyme R-10 (cellulase and pectinase) were incorporated into the procedure to digest partially the cell walls; this allowed dispersion of starch granules during slide preparation and also allowed the dye easier access into the cells.

Leaf tissue and thin sections of the periderm and outer parenchyma layers of white and green (4, 8 and 12 days of illumination) potato tissue were cut into 2 mm squares and suspended on separate aliquots of mannitol buffer (0.6 M-mannitol, 0.1 M-sodium citrate, 0.2 M-Na_2HPO_4, pH 5.7). These segments were then transferred to mannitol buffer containing 1% Macerozyme, and digestion of the cell walls was allowed for 2 h at 32° C. The partially digested tissue was washed several times in fresh mannitol buffer to remove the enzymes. The segments were placed in 4% (v/v) formaldehyde for 2 h for fixation and then washed in distilled water overnight at 4° C. The tissue segments were acid hydrolyzed for 12 min in 1 M-HCl at 60° C. After hydrolysis, the segments were washed in distilled water and then stained with Feulgen reagent for 2 h at 20° C. The segments were then washed in 3 changes of SO_2 water (0.022 M $K_2S_2O_5$, 0.05 M-HCl), 15 min in each, and rinsed in distilled water. The stained tissue was placed on a microscope slide and a drop of 25% (v/v) acetic acid was added. A silicon-coated coverslip (size number 2, Chance Propper Ltd., Warley, U.K.) was placed over the tissue and tapped firmly to spread the tissue underneath. Further spreading was achieved by placing several layers of filter paper over the coverslip and applying very firm pressure. The dispersal of tissue was checked by light microscopy (Zeiss, West Germany). The slide was then placed on solid CO_2 for 2-3 min, and the coverslip was then removed with a razor blade. The slide was placed in absolute ethanol to dehydrate the tissue, the ethanol was drained off and Euparal was added to the tissue to form a permanent mount with the coverslip (size number 0). The slide was turned upside down and blotted to remove air bubbles and any excess mounting material.

The fluorimetric measurements of the stained nuclei were performed on a Leitz MPV3 microscope photometer supplemented with a 200 W mercury lamp, BP 530-560 exciter filter (which allows only green light to pass through), RKP beam splitter mirror and a LP 580 suppression filter. Individual nuclei were analysed using a FL 40 objective and a PM-tube at a constant high voltage of 1.9 kV.

Two hundred nuclei were measured for each of the green and white potato tissues and 100 nuclei for leaf tissue. To correct for slight differences in background fluorescence, background readings were taken for each nucleus reading and subsequently subtracted to give the adjusted reading.

3.3 Growth of Bacteriophage λ and Subsequent DNA Extraction

Methods for growth of *E. coli* and bacteriophage λ, isolation of phage λ, and extraction of DNA from phage λ were adapted from methods in Cold Spring Harbor Laboratory Manuals (Davis, *et al.*, 1980; and Maniatis, *et al.*, 1982) and from Loenen and Brammar (1980).

3.3.1 Bacteriophage λ growth and amplification in host *E. coli* cells

An *E. coli* stock culture was prepared from the original solution of WL87 *E. coli*. A fresh overnight *E. coli* culture (10 ml), see below, was centrifuged for 10 min at 4000 g. The pellet was resuspended in 1 ml of 0.010 M-$MgSO_4$ and then two volumes of 80% (v/v) glycerol were added. The stock culture was stored at -20° C and used for future overnight *E. coli* culture preparations. These cultures remain viable for up to a few years.

Fresh overnight cultures of *E. coli* were made by inoculating a sterile loop of *E. coli* stock culture into 100 ml conical flasks containing 25 ml

L-broth [0.5% (w/v) bacteriophage λ, 2.5% (w/v) yeast extract, 0.043 M-NaCl, 0.003 M-glucose] and 0.025 g maltose. The L-broth/maltose/*E. coli* liquid medium was incubated overnight at 37° C, shaking.

Phage λ stock solutions were made using 10^0-10^6 fold dilution series of the concentrated stock (wild type) from W. Brammar. Aliquots of 10 µl from each of the dilutions, 50 µl fresh overnight *E. coli* culture, and 250 µl 0.010 M-$MgSO_4$ were incubated shaking from 15 min at 37° C. Then 3 ml of top agar [1.3% (w/v) nutrient broth, 0.65% (w/v) bacto-agar] were added. The mixture was immediately poured onto solidified bottom agar plates [1.3% (w/v) nutrient broth, 1.0% (w/v) bacto-agar].

Fifteen minutes were allowed for the top agar to solidify; then the plates were inverted and incubated for approximately 6 h. The plates showing confluent lysis within 6 h were incubated overnight at 4° C with 4 ml L-broth. The overlaid L-broth was drawn off, 1 drop $CHCl_3$ was added, and the mixture centrifuged at 20,000 g for 3 h at 4° C. The phage pellet was resuspended in 1 ml phage buffer [0.022 M-KH_2PO_4, 0.049 M-Na_2HPO_4, 0.085 M-NaCl, 1.000 M-$MgSO_4$, 0.100 M-CaCl, 1% (w/v) gelatine] by shaking overnight at 4° C. These phage stocks were stored at 4° C and were used in all subsequent dilution series for growing up phage λ for DNA extraction.

Growth of phage λ for DNA extraction was carried out as was the preparation of phage λ stock solutions. Using a serial dilution series it was found that a 10^4 fold dilution of stock phage λ was needed to yield a 10 µl sample of phage λ which showed confluent lysis after approximately 6 h on an *E. coli* lawn.

3.3.2 Bacteriophage λ DNA isolation

Phage particles were obtained from plates showing confluent lysis. To each plate, 1 ml phage buffer was added. Agar was inhibited from mixing with the overlay buffer by placing the plates, containing the buffer, in a 4° C cold room for 15-30 min. (Agar acts as a potent inhibitor of DNA enzymes, including endonucleases.) 100 µl of the overlay buffer, containing diffused λ particles, were mixed with 1 ml phage buffer containing a drop of $CHCl_3$. After spinning the solution in a microfuge (microCentaur, MSE) for a few seconds at the low setting to separate the $CHCl_3$ from the buffer, 100 µl of the buffer containing λ particles were added to 150 µl *E. coli* overnight culture and 0.01 M-$MgSO_4$ in a conical flask. This was left for 15 min at room temperature, which allowed the phage to be absorbed by the *E.coli* cells. To regrow the *E. coli* cells infected with phage, 200 ml L-broth containing 0.02 M-$MgSO_4$ were added. For lysis to occur, the mixture was incubated overnight at 37° C, shaking. A fully lysed *E. coli* culture showed considerable bacterial debris, which appeared as large stringy clumps. To the lysed culture, 20 ml l^{-1} of $CHCl_3$ were added and the culture was left shaking for 30 min at 37° C, then centrifuged for 10 min at 10,000 g. The dead *E. coli* cells and $CHCl_3$ were precipiated. The supernatant was removed and spun at 20,000 g for 3 h at 4 °C. A pellet of λ phage was obtained and resuspended in 1 ml phage buffer by shaking overnight at 4° C.

A 10 min 10,000 g centrifuge spin sedimented any remaining debris. The supernatant was treated with DNase I (10 µg ml^{-1}) and RNase-A (10 µg ml^{-1}) for 1 h at room temperature. The phage particles were then precipitated by spinning at 20,000 g for 3 h at 4° C. The pellet was resuspended in 1 ml TE buffer (Table 3.1) by shaking overnight at 4° C.

Protease (10 mg ml^{-1} stock solution in TE buffer predigested for 1 h at 37° C and stored at -20° C) was added to the resuspended pellet of phage to yield a final concentration of 1 mg ml^{-1}. The phage suspension was dialysed for 2 h at 37° C against pronase buffer [0.020 M-Tris-HCl (pH 7.5), 0.100 M-NaCl, 0.001 M-EDTA, 0.002% (v/v) triton X-100] and then placed in a glass centrifuge tube. The saturated phenol layer of crystalline phenol, melted in pronase buffer (60° C), was added to the protease digest to aid in the dissociation of the phage protein coat. DNA was extracted by gently rolling the solution, removing the aqueous layer and repeating by adding new phenol to the aqueous layer. Any remaining phenol was removed from the aqueous layer by dialysis against 500 ml pronase buffer (4 changes in 24 h).

Phage λ DNA was precipitated by adding 2 volumes of ethanol to 400 µl aliquot samples and then storing them for several hours at -20° C. The solutions were spun in a microfuge for 25 min at 11,000 g, and the supernatant was discarded. The resulting phage λ DNA pellet was resuspended in 20 µl TE buffer and stored at -20° C.

3.4 Extraction of DNA and RNA from Isolated Chloroplasts

Methods for chloroplast isolation and chloroplast DNA extractions were adapted from Kolodner and Tewari (1975) and Herrmann (1982). All procedures were carried out at 4° C.

3.4.1 Chloroplast isolation

Thirty grams of leaf tissue were homogenized in 120 ml of chilled buffer A [0.25 M-Tris-HCl_2(pH 7.5), 0.50 M-sucrose, 0.01 M-$MgCl_2$, 0.04 M-2-mercaptoethanol], by giving the mixture two 5 sec bursts in a Waring blender at maximum power. The homogenate was filtered through 3 layers of muslin and then centrifuged for 1 min at 400 g in a bench centrifuge,

giving a pellet of cell debris and nuclei. The supernatant was spun at 2,000 g for 10 min to pellet the chloroplasts. The supernatant was checked by phase contrast microscopy for intact chloroplasts; if necessary, centrifugation was repeated.

The pelleted chloroplasts were resuspended in 7 ml of buffer B [0.05 M-Tris-HCl (pH 8.0), 0.01 M-$MgCl_2$, 0.04 M-2-mercaptoethanol], purified through a continuous sucrose gradient (20-55% sucrose in buffer B, left overnight to stabilise) by carefully layering the chloroplast suspension on top of the gradient and spinning at 80,000 g (Beckman Ultracentrifuge, SW28 rotor head) for 35 min. During this step, any contaminating nuclei, nuclear fragments, multiorganelle particles, membrane-associated DNA and bacteria precipitated to the bottom. Two chloroplast bands were formed. According to the literature (Herrmann, 1982), the upper band contains broken chloroplasts and the lower band contains whole intact chloroplasts. When viewed by phase contrast microscopy, both bands contained intact chloroplasts, with greater intact chloroplast density found in the upper band. Thus in most cases, only the top band was taken, though in some instances both bands were combined. The extracted band(s) was then diluted with one equal quantity of buffer B and the chloroplasts were then reprecipitated with a 2,000 g spin for 10 min. This step should pellet the intact chloroplasts.

3.4.2 DNA extraction

The chloroplast pellet was resuspended in 1 ml of buffer B containing 5% sodium sarcosinate, to lyse the chloroplasts, and mixed thoroughly. To aid in lysis, the mixture was then frozen at -20° C for several hours. The mixture was thawed and a small sample was checked by phase contrast microscopy to verify that complete lysis had occurred.

It was found that large quantities of protein tended to obscure the DNA bands in a CsCl gradient, so phenol/$CHCl_3$ steps were introduced to deproteinize the DNA Sample. The saturated phenol layer of crystalline phenol melted in buffer B (60° C) was added to the lysed chloroplast solution and mixed thoroughly by inverting several times. The aqueous layer was extracted and the phenol step was repeated. Any remaining phenol was removed by adding $CHCl_3$:isoamyl alcohol (24:1) and after mixing well the aqueous layer was removed. The aqueous layer was mixed with 4 ml (1X) SSC (see Table 3.1) and 4.5 ml were added to 4.7 g CsCl. Ethidium bromide was added to give a final concentration of 10 μg ml^{-1}. This mixture was centrifuged for approximately 18 h at 230,000 g (Beckman Ultracentrifuge, VTi 65 rotor head).

Two DNA bands were clearly visible when the gradient was viewed in uv light. The eithidium bromide, interclated between the DNA bases, absorbed the uv light energy at 300 nm and then re-emitted the energy as fluorescence at 590 nm (Davis, *et al.*, 1980). The top band contained linear chloroplast DNA most likely contaminated with nuclear DNA, and the lower band contained closed circular DNA. The lower band only was always taken. It was collected by first releasing the pressure in the Beckman tube by cutting the seal, and then puncturing the side of the tube with a 21 or 23 gauge needle just under the fluorescent DNA band. The fluorescent band was slowly drawn out and placed in an Eppendorf tube. The ethidium bromide was removed by adding an equal volume of butan-1-ol to the DNA sample. After mixing, the pink-coloured butan-1-ol phase was removed and discarded. The extraction of ethidium bromide with butan-1-ol was repeated until all of the pink colour of the ethidium bromide was removed from the aqueous layer. The DNA solution was then either made up to 2 ml using TE buffer, placed in dialysis tubing and dialysed for 2 h against 1 ml TE buffer to remove any salt; or an

alternative preferred method for desalting using microconcentrators (Amicon Ltd., Stonehouse, U.K.) was used. In the latter method, 2 ml of the DNA/CsCl solution was put into the sample reservoir of the concentrator and spun at 5,000 g for approximately 1 h, or until 1 ml of the solution was left in the sample reservoir. Then 1 ml TE buffer was added to make the DNA solution up to 2 ml again and the sample was respun. This step was repeated twice to ensure that the DNA solution was desalted. After the final dilution the DNA solution was concentrated to about 100 ml and the filtrate was discarded.

After desalting using either method, the DNA was precipitated with ethanol, up to 0.8 M-NaCl and 2 volumes of 95% ethanol were added. To precipitate the chloroplast DNA, the samples were stored overnight at -20° C and then spun for 15 min at 11,000 g, maximum speed, in a microfuge. The supernatant was removed and the DNA pellet was resuspended in TE buffer.

3.4.3 RNA extraction

Since the large species of chloroplast rRNA is extremely sensitive to temperature increases above 4° C, all procedures for RNA extraction and purification must be carried out at 4° C (Leaver, 1982).

RNA from the lysed chloroplasts precipitated against the side of the tube during CsCl density gradient centrifugation. RNA, which also takes up ethidium bromide although not to the extent of DNA, fluoresced when excited with uv light. The RNA precipitate was easily collected by carefully emptying the tube of the CsCl/ethidium bromide solution, after the DNA had been removed, and resuspending the precipitate in a small quantity (1 ml) of sterile TE buffer. The ethidium bromide was removed (see above) and the RNA was mixed directly with 2 volumes of 95% ethanol, placed at -20° C overnight and precipitated by centrifugation at 11,000 g for 15 min in a microfuge. The RNA pellet was resuspended in TE buffer or sterile distilled water.

3.5 Digestion of DNA with Restriction Endonucleases and Separation of the Fragments by Agarose Gel Electrophoresis

Procedures for restriction enzyme digestions and agarose gel electrophoresis of the cleaved fragments were taken from Maniatis *et al.* (1982) and Davis *et al.* (1980).

3.5.1 Restriction endonuclease digestions

Digest reactions were made up using 1-2 µg of phage λ DNA or 5-10 µg of chloroplast DNA in volumes not exceeding 5 µl TE buffer. Twenty-five µl of 2 x restriction endonuclease buffer of either high salt concentration [0.1 M-NaCl, 0.05 M-Tris-HCl (pH 7.5), 0.01 M-$MgCl_2$, 0.001 M-DTT] for Sal I and Eco RI digestions or medium salt concentration [0.050 M-NaCl, 0.010 M-Tris-HCl (pH 7.5), 0.10 M-$MgCl_2$, 0.001 M-DDT] for Bam HI and Pst I digestions were added; using the lower salt concentration buffer for double-digestions. Sufficient restriction endonuclease was added to digest the quantity of DNA present. In principle, 1 unit of enzyme cleaves 1 µg of DNA in 15 min, but the purity of the DNA can influence the amount of enzyme required. Twice as much enzyme per µg DNA was used in chloroplast DNA digestions than in phage λ digestions. Sterile distilled water was added to make the total volume up to 50 µl and the reaction mixture was gently vortexed. The mixture was incubated for 3-4 hours at 37° C and the reaction terminated by addition of 20 µl stop buffer [5% (w/v) SDS, 25% (v/v) glycerol, 0.025% (w/v) bromophenol blue]. This mixture was then heated to 60° C for 5 min to inactivate any contaminating enzymes and to melt reannealed cohesive ends.

3.5.2 Agarose gel electrophoresis of DNA fragments

Restriction digests of DNA were run on either long slab gels (20 cm x 10 cm) or on mini gels (10 cm x 8 cm; Uniscience Ltd., Cambridge, U.K.). The slab gel was 0.7% agarose in acetate buffer [0.050 M-Tris-acetate

(pH 7.9), 0.020 M-sodium acetate, 0.001 M-EDTA, 0.010 M-NaCl) and was run in the non-submarine mode using filter paper and tissues as wicks connecting the gel to the two troughs containing acetate buffer and opposing electrical currents (see Maniatis *et al.*, 1982, pp.154-155). The mini gel was 0.7% agarose in TBE buffer (see Table 3.1), run in the submarine mode with TBE buffer to overlay the gel.

For both gels, sufficient restriction enzyme digest was carefully pipetted into each well. Electrophoresis was started at a high voltage (3 V cm^{-1} for the slab gel, 100 V for the mini gel) for 15 min, to move the DNA sample into the gel. The voltage was then reduced (1.5V cm^{-1} for the slab gel and 70-90 V for the mini gel), and the slab gel was run for 18-24 h and the mini gel for 45 min. After electrophoresis, the gels were stained with ethidium bromide (0.5 µg ml^{-1} gel buffer) for 15 min and viewed under uv light.

3.6 Cloned pBR322 DNA Amplification and Extraction

The procedure for the transformation of *E. coli* HB101 cells with plasmid was from Mandel and Higa (1970). The plasmid was pBR322 containing a cloned wheat chloroplast DNA insert, either the sixth PstI-fragment (P_6) or the second Bam HI fragment (B_2). Amplification of the plasmid was achieved by following the method described by Maniatis *et al.* (1982), which is based on the ampicillin or tetracycline resistance of pBR322 being altered by the insertion of foreign DNA fragments into the plasmid DNA; *i.e.*, plasmids with a PstI restriction site insert (pBR322-P_6) only convey tetracycline resistance and plasmids with a Bam HI restriction site insert (pBR322-B_2) only convey ampicillin resistance. The procedure for plasmid DNA extraction was adapted from Davis *et al.* (1980).

3.6.1 Plasmid amplification and selection

A fresh overnight bacterial culture was made by inoculating 25 ml L-broth with a loopful of *E. coli* HB101 from a slope. This was grown up overnight at 37° C. Bacterial cells were then grown to a density of approximately 5 x 10^7 cells per ml, by inoculating 100 ml of L-broth in a 500 ml flask with 1 ml of the overnight bacterial culture, vigorously shaking at 37° C until the absorbance of the culture reached 0.5 at 600 nm. This typically took between 2-3 h. The culture was chilled on ice for 10 min and then centrifuged at 4,000 g for 5 min at 4° C to pellet the bacterial cells. The supernatant was discarded and the cells re-suspended in 50 ml of ice-cold, 0.05 M-$CaCl_2$ and 0.01 M-Tris—HCl (pH 8.0). The cell suspension was put on ice for 15 min and was then centrifuged at 4,000 g for 5 min at 4° C. The supernatant was discarded and the pellet of cells was again resuspended in 7 ml of ice-cold, 0.05 M-$CaCl_2$ and 0.01 M-Tris-HCl (pH 8.0). Aliquots of 200 µl were dispensed into pre-chilled Eppendorf tubes and these were stored for 12-24 h at 4° C. During this 12-24 h period, the efficiency of transformation increases four- to six-fold, due to the action of $CaCl_2$ permeating the bacterial cell membrane (Dagert and Ehrlich, 1979); longer incubations will result in lowered efficiency.

Less than 40 ng of pBR322, either pBR322-P_6 or pBR322-B_2, was added to the 200 µl aliquots of suspended bacterial cells. The samples were transferred to a water bath, pre-heated to 42° C, for 2 min. One ml L-broth was added to each tube and they were then incubated at 37° C for 30 min (tetracycline selection, pBR322-P_6) or 1 h (ampicillin selection, pBR322-B_2) without shaking. This period allowed the bacteria to recover and to begin to express antibiotic resistance.

Plates were made up with 12.5-15 µg tetracycline [from a stock of 12.5-15 mg ml^{-1} tetracycline in ethanol/water (50%), (v/v); sterilised by filtration and stored covered at -20° C in aliquots] per ml bottom agar (see Section 3.3.1) for growth of *E. coli* transformed with the pBR322-P_6 with 50 µg ampicillin (from a stock of 5 mg ml^{-1} in water; sterilised by filtration, stored at -20° C in aliquots) per ml bottom agar for growth of *E. coli* transformed with the pBR322-B_2. Due to the light sensitivity of tetracycline, all solutions containing tetracycline were kept covered.

If the selection was for tetracycline resistance (pBR322-P_6) the entire transformation mixture was mixed with 3 ml top agar (see Section 3.3.1) and vortexed briefly to ensure that the contents were well mixed and that no clumps of bacteria remained. This was then poured onto a plate containing bottom agar and tetracycline. The plate was swirled to obtain an even spread of the mixture over the bottom agar. The top agar was allowed to set and then the plates were incubated, inverted and covered, for 12-16 h at 37° C.

The same procedure was used for selection of bacterial cells having ampicillin resistance (pBR322-B_2), except several dilutions were made before adding infected bacteria to the top agar to reduce the cell density per plate. With ampicillin resistance, it has been noted that the number of transformations obtained does not increase in linear proportion to the volume applied to the plate (Maniatis *et al.*, 1982). A possible reason for this may be that the cells killed by the antibiotic produce a toxic substance which kills nearby colonies. Plates containing ampicillin were incubated, inverted, at 37° C for 12-16 h.

A single colony was picked and placed into 10 ml L-broth with the appropriate antibiotic. This was then incubated overnight with vigorous shaking. L-broth (25 ml) containing the appropriate antibiotic in a 100 ml flask was inoculated with 100 µl of the overnight culture. This was incubated at 37° C with vigorous shaking until the culture reached late log phase, $Abs_{600} \simeq 0.6$. Twenty-five ml of the late log phase was used to inoculate 500 ml L-broth, pre-warmed to 37° C, containing the appropriate antibiotic in a 2 l flask. The mixture was incubated for exactly 2.5 h at 37° C with vigorous shaking, resulting in an Abs_{600} of approximately 0.4. Twenty-five ml of a solution of chloramphenicol (34 mg/ml in ethanol) was added to give a final concentration of chloramphenicol in culture of 170 µg ml^{-1}. This was incubated at 37° C with vigorous shaking for 12-16 h. Chloramphenicol blocks protein synthesis and thereby *E. coli* chromosomal replication ceases, because of the need for continued protein synthesis. However, it has no effect on plasmid replication.

3.6.2 Plasmid DNA extraction

The bacterial cells were sedimented at 4,000 g for 10 min at 4° C and then resuspended in 125 ml of 0.01 M-Tris-HCl (pH 8.5) and 0.001 M-EDTA, and resedimented. The bacterial cells were resuspended in 2 ml of 15% sucrose, 0.05 M-Tris-HCl (pH 8.5), 0.05 M-EDTA and 1 mg ml^{-1} freshly prepared lysozyme, transferred to a 10 ml centrifuge tube and incubated at room temperature for 10-60 min. To ensure lysis, 2 ml triton solution [0.1% (v/v) triton-X-100, 0.05 M-Tris-HCl (pH 8.5), 0.05 M-EDTA] were added, mixed well and the suspension was incubated at room temperature for 10-20 min. When lysis had occurred the suspension became very viscous. The lysed suspension was spun at 70,000 g in a SW27 rotor (Beckman Ultracentrifuge) for 1 h at 4° C. The supernatant was decanted into a graduated test tube, leaving the very viscous material above the

pellet. The volume was adjusted to 4 ml, 3.7 g solid CsCl and 400 µl of 10 mg ml^{-1} ethidium bromide were added. This was centrifuged in a VTi 65 rotor (Beckman Ultracentrifuge) at 220,000 g overnight. When viewed under uv light fluorescent bands were revealed, the upper (less dense) band consisted of contaminating *E. coli* chromosomal DNA and linear and broken plasmid DNA, and the lower (more dense) band contained the covalently closed circular plasmid DNA. The lower band only was collected, as described in Section 3.4.2.

3.7 Selection, Amplification and Extraction of Cloned Coliphage M13 Replicative Form (RF) DNA

Procedures for the transfection of *E. coli* with the coliphage M13, with the subsequent extraction of the amplified M13 DNA were taken from Davis *et al.* (1980) and Maniatis *et al.* (1982). A detailed description of the M13 vector system is presented by Messing (1983).

3.7.1 Transfection of *E. coli* JM107 with M13 clones

The *E. coli* JM107 was provided as a Bacti-disk - a gelatin based solid disk containing skim milk, ascorbic acid, charcoal and the *E. coli* strain. The disk was dissolved in 500 µl L-broth for approximately 10 min, and then streaked onto glucose/minimal media plates [0.6% (w/v) Na_2HPO_4, 0.3% (w/v) KH_2PO_4, 0.05% (w/v) NaCl, 0.1% (w/v) NH_4Cl, 0.001 M-$MgSO_4$, 0.0001 M-$CaCl_2$, 0.2% (w/v) glucose and 0.001% (w/v) thiamine]. The plates were incubated for 1-2 days at 37° C. This step was necessary to ensure that the *E. coli* retained its F episome on which is located the defective β-galactosidase gene. The remaining *E. coli* JM 107 suspension was made into a more permanent stock by adding an equal volume of sterile 80% glycerol and storing at -20° C.

Usually after two days *E. coli* JM107 colonies appeared on the minimal media plates. A colony was picked and grown up overnight in 2 x YT broth [1.6% (w/v) bactotryptone, 1.0% (w/v) yeast extract and 0.5% (w/v) NaCl] at 37° C, standing. A mid-log phase culture was prepared by a 1.5 h incubation at 37° C of 25 ml 2 x YT broth inoculated with 2 ml JM107 overnight culture. The bacterial cells were pelleted by centrifugation for 5 min at 2,000 g. The pellet was washed with 10 ml 0.1 M-$MgCl_2$ and reprecipitated. The pellet was then resuspended in 1.25 ml 0.1 M-$CaCl_2$ and incubated on ice for 20 min. Transfection with serial dilutions of the M13 vector plus insert (α-α subunit of the ATP synthase gene, β-β subunit of the ATP synthase gene, C-cytochrome f gene) was carried out on ice for 20 min, followed by 5 min at 42° C. Three ml YT top agar [1% (w/v) bactotryptone, 0.5% (w/v) NaCl, 0.5% (w/v) agar], 50 µl X-gal (from a stock of 20 mg X-gal ml^{-1} dimethyl formamide), and 10 µl IPTG (from a stock of 24 mg IPTG ml^{-1} distilled water) were added to the transfected bacterial cells. This was mixed and quickly poured onto solidified TA plates [1% (w/v) bactotryptone, 0.5% (w/v) NaCl, 1.5% (w/v) agar] before the top agar cooled, and incubated at 37° C overnight. Transformed cells containing the M13 vector were identified by a blue plaque, which resulted from the action of the defective host β-galactosidase gene and the complement M13 phage β-galactosidase gene fragment acting together to cleave X-gal to produce the blue dibromo-dichloroindigo by the induction of IPTG. Transformed cells containing the M13 vector plus the insert were identified by turbid white plaques, which resulted due to the M13 β-galactosidase gene fragment being disrupted by the inserted DNA, thus making β-galactosidase complementation impossible.

3.7.2 Isolation of the cloned M13 RF DNA

Four 10 ml aliquots of 2 x YT were incubated with a loopful of JM107 overnight culture. Into three cultures a white plaque, containing M13 plus insert (α, β, or C) DNA was placed. These were grown overnight at 37° C, standing. 0.25 ml of the uninfected culture was added to 10 ml aliquots of 2 x YT broth and shaken rapidly for 1 h. Then 0.125 ml infected culture was added to one of the uninfected cultures, and shaken rapidly for 4.5 h.

The infected cultures were spun at 2,000 g for 5 min to collect the bacterial cells. The cells were resuspended in 250 µl of 25% (w/v) sucrose, 0.05 M-Tris-HCl (pH 8.0) and placed in a 1.5 ml Eppendorf tube. Fifty µl of 10 mg ml^{-1} lysozyme in distilled water was added and the suspensions were kept at room temperature for 5 min. Then 2 µl of ice-cold 0.5 M-EDTA, pH 8.0, was added and kept at room temperature for 5 min. To ensure complete lysis of the bacterial cells, 250 µl triton lysis mixture [1% triton-X-100, 0.050 M-Tris-HCl (pH 8.0), 0.063 M-EDTA] were added and incubated on ice for 10 min. To pellet the chromosomal DNA and cellular debris, the suspensions were spun in a microfuge for 15 min. This step was repeated, due to the large quantities of chromosomal DNA and debris obtained. The supernatant containing the RF DNA was removed and 50 µl 2M-ammonium acetate were added. The RF DNA was phenol extracted (see Section 3.4.2) twice to rid the sample of proteins. Two volumes of ethanol were added to the DNA sample and it was stored overnight at -20° C. The RF DNA pellet was obtained by centrifugation at 11,000 g for 15 min. The pellet was made up in distilled water and stored at -20° C.

3.8 Production of Radiolabelled Probes by Nick-translation

Methods for DNA nick-translation were according to the recommended procedures supplied with the Nick-translation kit (BRL). Separation of radiolabelled nucleotide from radiolabelled DNA by gel filtration was adapted from Chelm (1982).

3.8.1 Nick-translation of DNA molecules

Three different DNAs were radiolabelled by nick-translation: (1) potato leaf chloroplast DNA, (2) pBR322 DNA plus a cloned wheat chloroplast DNA insert, and (3) DNA of M13 phage plus a cloned wheat chloroplast DNA insert. The procedure was similar for all of them.

The chloroplast DNA was radiolabelled with ^{3}H-TTP, using approximately 8 µCi (296 KBq) of ^{3}H-TTP (specific activity, 1.85 TBq mmol^{-1}) per µg of chloroplast DNA. M13 and pBR322 DNA's were labelled with ^{35}S-CTP (specific activity, 24 TBq mmol^{-1}) using approximately 40 µCi (1.48 MBq) of ^{35}S-CTP per 80 ng DNA.

^{3}H-TTP was packaged in an aqueous ethanol solution (1:1, v/v), which could not be used in the nick-translation reaction mixture. So the required amount of radiolabel was taken and placed in an Eppendorf tube, and the ethanol solution was removed by directing a gentle stream of nitrogen gas onto the surface of the solution. The ^{35}S-CTP was packaged in an aqueous solution (0.020 M-DTT) and therefore did not require this step.

Into the Eppendorf tube which contained the desiccated ^{3}H-TTP or the required amount of ^{35}S-CTP was placed 5 µl of solution A (Table 2.1), the DNA to be radiolabelled (made up to a volume of no greater than 5 µl with TE), and enough sterile distilled water to make the volume up to 45 µl. Then 5 µl of solution C (Table 2.1), containing DNAseI and

DNA polymerase I were gently, but thoroughly, mixed in. The reaction mixture was incubated for 60 min at 15° C, then terminated by the addition of 5 µl solution D (Table 2.1). Radiolabel incorporation was found to increase by increasing the amount of unlabelled nucleotides and DNaseI/DNA polymerase I added by 1.5 times.

3.8.2 Separation of DNA from residual nucleotides by Sephadex gel filtration

DNA molecules were separated from residual nucleotides by gel filtration, using a 80 cm Sephadex (G-50, fine) column equilibrated in TE buffer (pH 8.0). The column was run at 4 drops per min with 250 µl fractions being collected every min. Dextran blue and methylene blue were run previous to the DNA sample as markers to indicate the approximate DNA elution volume and the void volume (nucleotide elution), respectively.

3.8.3 Detection of the radiolabelled DNA peak by scintillation counting

The specific activity of the radiolabelled DNA was determined by scintillation counting (Packard, Minaxi Tri-carb 4000 series or LKB 1217 Rackbeta). A 2µl aliquot was taken from each of the fractions collected and 2 ml scintillation cocktail (0.5% PPO, 30% triton-X-100, 70% toluene) were added. This was vortexed briefly and then counted for 2 min, using the appropriate tritium or ^{35}S channel.

3.9 Extraction of Total DNA and Total RNA from Potato Tissues

Total DNA (nuclear, mitochondrial, and plastid) and total RNA (cytoplasmic and plastid) from potato leaf and potato tuber (white and green) tissues were obtained using a phenol/$CHCl_3$/isoamyl alcohol extraction procedure. Three solutions; 120 ml nucleic acid extraction buffer [0.100 M-Tris-HCl (pH 7.5), 0.005 M-EDTA, 0.400 M-NaCl, 0.5% (v/v) sodium sarkosinate], 60 ml phenol (AR grade) freshly equilibrated in nucleic acid extraction buffer, and 60 ml $CHCl_3$/isoamyl alcohol

(24:1) were thoroughly mixed and allowed to separate into two phases at 4° C. The aqueous phase was added to 30 g of potato tissue, taking only the periderm and outer layer of parenchyma tissue from tuber tissue, which was then homogenized in a Waring blender. The phenol/$CHCl_3$/isoamyl alcohol phase was added to the homogenate and stirred for 15 min at 4° C. The aqueous and phenol/$CHCl_3$/isoamyl alcohol phases were separated by centrifugation at 500 g for 5 min. The aqueous phase and the interphase were removed, mixed with an equal volume of phenol (freshly equilibrated with nucleic acid extraction buffer) and were stirred for 15 min at 4° C. The phases were separated by centrifugation at 500 g for 5 min and the aqueous phase and interphase removed. The phenol step was then repeated twice and the final aqueous phase was added to an equal volume of $CHCl_3$/isoamyl alcohol (24:1) and stirred for 15 min at 4° C. The aqueous phase was separated by centrifugation at 500 g for 5 min, removed and made up with 2 volumes of 95% ethanol. This was stored overnight at -20° C (or a few hours at -70° C) and then centrifuged at 3,000 g to precipitate the total DNA and total RNA.

The ethanol precipitated total DNA and total RNA pellet was resuspended in 1 ml TE buffer and run on a CsCl/ethidium bromide density gradient (see Sections 3.4.2 and 3.4.3) to separate the total DNAs and total RNAs. Both DNA bands were collected to give a total DNA sample, and the sample was desalted as described in Section 3.4.2. The total RNA pellet was collected as before (Section 3.4.3). Both total DNA and total RNA samples were ethanol precipitated, resuspended in TE buffer and stored at -20° C until needed.

3.10 Quantification of DNA

The concentration of DNA in a sample was determined by measuring the absorbance of the solution at 260 nm. An absorbance of 1 corresponds to approximately 50 μg ml^{-1} double-stranded DNA. For dot hybridisations, where DNA concentration is critical, the concentration of DNA samples were also estimated by diphenylamine assay (Burton, 1956), which is described below.

Duplicates of a dilution series of six salmon testes sperm DNA concentrations (1, 2, 5, 10, 15, 20 μg) and duplicate aliquots of potato tissue DNA (2-4 μg DNA) were made up in 10% PCA to a final volume of 500 μl in an Eppendorf tube. A 500 μl sample of 10% PCA, containing no DNA, was used as a blank. An equal volume of freshly made diphenylamine reagent (4 g diphenylamine dissolved in 100 ml glacial acetic acid and 1 ml H_2SO_4) was added to each sample. To this 25 μl acetaldehyde was added, and mixed by inverting the tube several times. The reaction was incubated overnight at 30° C.

After incubation, the DNA standards (salmon testes sperm DNA) and the potato tissue DNA samples were analysed spectrophotometrically against the blank at 595 and 700 nm. Using the corrected absorbance value (Abs_{595nm}-Abs_{700nm}), a standard curve was fitted to the salmon sperm DNA results, and from this potato tissue DNA concentrations were calculated.

Due to its toxic properties, preparation and analysis of solutions containing diphenylamine were carried out in a fume hood.

3.11 DNA-DNA Reassociation Reactions

The procedure used for analysing the percentage ptDNA in the total DNA population was adapted from Chelm (1982).

3.11.1 Denaturation and reassociation

The radiolabelled chloroplast DNA probe, 0.5 µg DNA (1×10^6 cpm μg^{-1} DNA) and 50 µg 'driver' DNA, sonicated on ice for 6.5 min at 50 W using a 45 nm probe (Sonicator-Rapidis 180, Ultrasonics Ltd., Shipley, W. Yorkshire, England) to yield 500-1000 bp fragments as determined by agarose gel electrophoresis, were made up to 1 ml with reassociation buffer [0.025 M-Tris-HCl (pH 7.4), 0.300 M-NaCl, 0.001 M-EDTA]. Two control 40 µl samples were taken and frozen, at -20° C, for later assay as the total radiolabelled double-stranded standard. The remaining DNA solution was denatured by heating at 100° C for 10-15 min, and two 40 µl samples were immediately taken and frozen, at -20° C, for the total radiolabelled single-stranded standards. The denatured DNA solution was then transferred to a 60° C water bath and 40 µl samples were taken at specific time intervals and immediately frozen in dry ice.

3.11.2 Duplex DNA isolation and analysis

Single-stranded DNA in each 40 µl sample was digested to 5'-mononucleotides by the addition of 20 units of S_1 nuclease in S_1 nuclease buffer (0.100 M-NaCl, 0.025 M-Na acetate, 0.010 M-$MgCl_2$, 0.0001 M-$ZnCl_2$, 0.005 M-2-mercaptoethanol). Two µg of denatured calf thymus DNA were added as carrier, to give a total volume of 150 µl. This reaction mixture was incubated for 2 h at 37° C.

Duplicates of 50 µl from each time point sample were spotted on 2.3 cm DE-81 filter discs (Whatman Ltd., Kent, U.K.), previously soaked in phosphate buffer [0.24 M-NaH_2PO_4, 0.24 M-Na_2HPO_4 (pH 6.8)] and dried completely. The discs containing the DNA samples were partially dried

for 30 min, then washed three times in a large volume of phosphate buffer to remove nucleotides, and then dried thoroughly. The discs were placed in scintillation vials, with 2 ml of scintillant (0.5% PPO in toluene), and counted for 10 min using the tritium channel [LKB Instruments (UK) Ltd.].

3.12 DNA-DNA and RNA-DNA Dot Blot Membrane Hybridisations

The DNA-DNA and RNA-DNA dot hybridisation methods were adapted from the recommended procedures provided with the Pall Biodyne nylon membranes.

3.12.1 DNA-DNA dot hybridisations

3.12.1.1 Membrane preparation and DNA binding.-Pall Biodyne transfer membranes (Pall Ultra-fine Filtration Corporation, Glen Cove, N.Y., U.S.A.) were prewet before use. The membranes were soaked in distilled water for a few seconds and then transferred to 15 x SSC (Table 3.1) for 30 min.

Dilution series, to yield 0.2, 0.1, 0.05, 0.025 µg total DNA per dot, of leaf, green tuber (4 and 8 days of illumination) and white tuber tissue total DNA were made up to equal volumes with sterile distilled water, each dilution containing enough DNA to perform four replicates. The total DNA samples were denatured by adding an equal volume of 4 M-NaOH and then neutralised after 15 min with 2 M-acetic acid. The volume was made up to the required DNA concentration with 20 x SSC to give a final solution of 15 x SSC. The samples were applied to the membrane through a hybri-dot manifold (Schleicher and Schnell, Dassel, West Germany) which was under light vacuum.

Unlabelled probe DNA was also applied to the membrane and used as a standard. Before application, the circular molecules of the pBR322 plus insert DNA and the M13 plus insert DNA were sheared by passing the DNA through an 18-gauge hypodermic needle (10 times), and the DNA was

then denatured as above. The unlabelled probe DNA was added in a dilution series to yield a corresponding level hybridisation to the other samples.

After spotting the required amount of DNA onto the membrane, the individual sample wells were rinsed with 15 x SSC. The membrane was air dried for 30 min and then baked in a vacuum oven at 80° C for 1-2 h. The membrane could then be stored *in vacuo* at room temperature until needed.

3.12.1.2 DNA-DNA prehybridisation.-Salmon testes sperm DNA was used as non-homologous DNA, to bind to non-specific sites on the membrane in a prehybridisation step. A stock solution of 10 mg ml^{-1} was prepared and sonicated for 15-25 min. This stock was kept frozen at -20° C until needed.

For each membrane, 4 ml of hybridisation solution was used. The hybridisation solution contained 5 x Denhardt's buffer (from 50 x Denhardt's stock, Table 3.1), 5 x SSPE (from 20 x SSPE stock, Table 3.1), 0.2% (w/v) SDS, and 500 µg ml^{-1} denatured salmon tests sperm DNA (15 min at 100° C). The membrane was heat-sealed in a plastic bag with hybridisation solution, and incubated at 65° C for 3-4 h.

3.12.1.3 DNA-DNA hybridisation.-After prehybridisation, the hybridisation solution was removed. Approximately 80 ng (1-10 x 10^8 cpm μg^{-1} DNA) ^{35}S-CTP labelled pBR322 plus insert DNA was combined with salmon testes sperm DNA (250 µg ml^{-1} hybridisation solution) and denatured at 100° C for 15 min. The denatured DNAs were added to new hybridisation solution to make 4 ml. This radioactive solution was added to the membrane in a plastic bag, the bag was sealed and then sealed in another bag. Hybridisation took place overnight (approximately 17-24 h) at 65° C.

3.12.1.4 Procedure for stringent washing of hybridisation membranes.- After hybridisation, the radioactive hybridisation solution was properly disposed of and the membrane was dipped in wash buffer [0.005 M-Na phosphate, 0.001 M-EDTA, 0.2% (w/v) SDS (pH 7.0)] with 2 x SSC and 0.1% (w/v) SDS. The membrane was then placed in a plastic bag with 250 ml wash buffer and shaken vigorously (200 rpm, Gallenkamp Orbital incubator) at room temperature for 5 min. This step was repeated with fresh buffer three times. The membrane was then washed in 250 ml wash buffer containing 0.1% (v/v) SSC and 0.1% (w/v) SDS at 50° C for 15 min. This step was repeated once. The membrane was removed from the bag containing the buffer, wrapped in cling film and autoradiographed using Kodak X-OMAT S, 18 x 24 cm, film (Kodak Ltd., London, U.K.). The exposed film was developed using Kodak D-19 developer for 5 min, fixed using Kodak Unifix powder for 5 min, and then rinsed in distilled water.

3.12.2 RNA-DNA dot hybridisations

The method for RNA-DNA hybridisations, using radiolabelled pBR322-B_2 or pBR322-P_6 probes and radiolabelled M13-α, M13-β, or M13-C probes was slightly different from DNA-DNA hybridisations.

Total RNA samples [leaf, green tuber (4 and 8 days of illumination), and white tuber tissues] were spotted directly onto the membrane without the prewetting and denaturation steps. Total RNA was added at higher concentrations - dilution series: 2.0, 1.0, 0.5, 0.25 µg total RNA per dot - and in a volume less than 5 µl, under a light vacuum. The membrane was baked in a vacuum oven as above.

Prehybridisation was carried out as in the DNA hybridisations, except that the hybridisation solution was altered. The solution con-

tained: 5 x Denhardt's, 5 x SSC, 0.050 M-Na phosphate (pH 6.5), 0.1% SDS, 250 μg ml^{-1} denatured salmon testes sperm DNA and 50% (v/v) formamide. The incubation was carried out at 42° C, instead of 65° C as for the DNA-DNA hybridisations, due to the presence of formamide.

Hybridisation of the DNA probe to the total RNA samples was carried out using the altered hybridisation solution with one of the four denatured radiolabelled probes. The M13 probes were labelled to a specific activity of 10^7 cpm μg^{-1} DNA, and 80 μg were used for each membrane. Hybridisation occurred at 42° C overnight (17-24 h).

The washing procedure was the same as used for the DNA-DNA hybridisations.

3.13 High-Resolution Separation of Ribosomal RNA (rRNA) by Polyacrylamide Gel Electrophoresis

The dilute polyacrylamide tube gel system described here was developed by Loening (1967).

For preparation of 2.4% acrylamide tube gels, 1.6 ml of acrylamide stock [15% (w/v) acrylamide and 0.75% (w/v) bisacrylamide, filtered and stored covered at 4° C], 3.3 ml BE buffer [0.006 M-EDTA, 0.12 M-Tris-HCl (pH 7.8), 0.01 M-sodium acetate] and 5.1 ml distilled water were mixed together. This solution was degassed and 10 μl TEMED and 100 μl freshly prepared ammonium persulphate (100 mg ml^{-1}) were added. The solutions were mixed, avoiding excessive aeration, and rapidly pipetted into silica glass, vertical tubes (10 cm x 4 cm) held upright in a stand that prevented leakage of the polyacrylamide solution from the bottom. Water was carefully layered on top to ensure a flat gel surface and to prevent exposure to the air, and the gel allowed to polymerise for 1-2 h at room temperature.

Thirty µg of total RNA in 10 µl TE buffer, extracted as described in Section 3.9, were added to 10 µl solubilisation buffer [0.073 M-Tris-HCl (pH 7.8), 0.004 M-EDTA, 0.066 M-sodium acetate, 20% (w/v) SDS]. To this 10 µl stop buffer [5% (w/v) SDS, 25% (v/v) glycerol, 0.025% (w/v) bromophenol blue] were added. Before adding the total RNA sample to the gel, the tube gels were pre-electrophoresed at 4 mA per gel for 30 min in running buffer [0.002 M-Na-EDTA, 0.04 M-Tris-HCl (pH 7.8), 0.033 M-NaAc, 0.05% (w/v) SDS]. The total RNA samples were then added to the gel surface. The gels were run at 4° C overnight at 1-2 mA (approximately 1 V) or 8 h at 4 mA. The positions of the rRNA bands were detected by scanning the gel at 254 nm in a MSE 1310 gel scanner with an ISCO UA-5 absorbance/fluorescence monitor, and a LDC/Milton Roy C1-10 integrator and graph plotter.

3.14 Hybrid-Release Translation

The method for selecting ctRNA and then translating the mRNA into protein products, hybrid-release translation, was from Maniatis *et al.* (1982). Fluorography of the protein products was adapted from Bonner and Laskey(1974).

3.14.1 Binding chloroplast DNA to Biodyne-A nylon membranes

Purified chloroplast DNA, 20 µg, was made up to 20 µl in distilled water, vortexed briefly and then denatured by heating for 10 min at 100° C. The DNA solution was quickly chilled on ice and an equal volume of 1 M-NaOH was added. This was incubated at room temperature for 20 min.

The DNA solution was neutralised by adding 0.5 volumes of a solution of 1 M-NaCl, 0.3 M-Na citrate, 0.5 M-Tris-HCl (pH 8.0), and 1 M-HCl. This was mixed well and immediately chilled on ice.

Pall Biodyne-A nylon membranes were cut into 3 mm squares and placed on a Millipore filter holder [Millipore (U.K.), London]. Light vacuum pressure was applied to the Millipore apparatus and 5 µl of the denatured DNA solution spotted onto the membrane. Once absorbed, another 5 µl was added until 20 µg of DNA had been added. The membrane was allowed to air dry for 1 h and then it was placed into a sterile 50 ml, screw-capped, conical tube. The membrane was washed twice with 50 ml 6 x SSC at room temperature, and allowed to dry for 1 h. The membrane was then placed in a test tube with a loose metal cap and baked for 2 h at 80° C in a vacuum oven. The DNA-bound membrane could then be stored at room temperature *in vacuo* until needed.

3.14.2 Hybridisation and elution of mRNAs

The membrane with bound ctDNA was placed in a silicon-coated vial with 1 ml water and heated in a boiling water bath for 1 min, cooled on ice, and the water was removed by aspiration. Another ml of water was added, the vial vortexed briefly and the water was removed by aspiration. These two steps ensured that any loosely bound chloroplast DNA was eluted and removed.

One ml of hybridisation solution [65% (v/v) formamide, 0.020 M-1,4-piperazinediethane sulphonic acid (PIPES; pH 6.4), 0.2% (w/v) SDS, 0.4 M-NaCl, 100 µl ml^{-1} tRNA, 500 µg enriched chloroplast RNA (see Section 3.4.3), and the appropriate amount of RNAase inhibitor] was heated at 70° C for 10 min, added to the membrane, and incubated for 3 h at 50° C.

Following hybridisation, the solution was removed by aspiration and 1 ml of wash solution [0.010 M-Tris-HCl (pH 7.6), 0.15 M-NaCl, 0.001 M-EDTA, 0.5% (w/v) SDS], preheated to 65° C and maintained at 65° C throughout the washing procedure, was added to the membrane. This was vortexed

for several seconds and the wash solution was removed by aspiration. The wash step was repeated nine times. The membrane was then washed twice with wash solution minus the SDS. The membrane was transferred to a silicon-coated Eppendorf tube, and 300 µl water and 30 µg tRNA were added. The tube was placed in a boiling water bath for 1 min and then quickly transferred to a solid CO_2/ethanol bath, in a snap-freeze step which released the hybridised RNA. The sample was slowly thawed on ice and the membrane was removed. The eluted RNA solution was extracted with an equal volume of phenol/$CHCl_3$ and then precipitated by adding 60 µl 2 M-Na acetate (pH 5.2) and 1 ml ethanol. The tube was placed in solid CO_2/ethanol for 20 min and the RNA was recovered by centrifugation in a microfuge at 11,000 g for 10 min. The ethanol was removed and the RNA pellet was dried in a vacuum desiccator and resuspended in 5 µl water.

3.14.3 *In vitro* protein synthesis

Approximately 30 µCi (1.1 MBq) of ^{35}S-methionine were 'blown down' with nitrogen gas. Into this tube the following reaction mixture was placed: 35 µl rabbit reticulocyte lysate, 2.5 µl 2 M-K acetate, 2.5 µl amino acid mixture minus methionine and 10 µl of RNA solution. Typically 4 reaction mixtures were made: (1) a control with no RNA added, just 10 µl of water; (2) a leaf total RNA sample using 30 µg total RNA in 10 µl distilled water; (3) a chloroplast-enriched RNA sample using 30 µg ctRNA in 10 µl distilled water; and (4) combined samples of the hybrid-release RNA in 10 µl distilled water. The reaction mixtures were incubated at 30° C in a water bath. Samples of 5 µl were taken at 0, 5, 10, 20 and 40 min intervals and added to 500 µl 1 M-NaOH with 10% H_2O_2. These were placed in a water bath at 37° C for 10 min to hydrolyse the aminoacyl tRNAs and then put on ice. The time point samples were made to 2 ml with ice cold

25% (w/v) TCA and 0.1% (w/v) BSA to precipitate the proteins. They were left on ice for 30 min and precipitated at 2000 g for 10 min. The protein pellet was washed twice with cold 5% TCA and then resuspended in 1 ml 1 M-NaOH. Half of this was neutralised with acetic acid and then an equal volume of water was added. Five ml scintillant were added, vortexed briefly and counted to determine the amount of radiolabel incorporation in the TCA precipitable protein products.

3.14.4 SDS-Polyacrylamide gel electrophoresis of the protein products

The remainder of the translation mixture not used for TCA precipitation, 25 µl, was added to a SDS-polyacrylamide gel for qualitative analysis of the protein products.

The SDS-polyacrylamide gel consisted of a 7.4% stacking gel and a 10% separating gel. The separating gel was prepared using 13.5 ml stock acrylamide (22.2% acrylamide, 0.6% bisacrylamide in water), 15 ml gel buffer [0.05 M-Tris-HCl (pH 8.0), 0.6% SDS], 15 ml freshly prepared ammonium persulphate (15 mg ml^{-1}), and 45 µl TEMED. The separating gel was poured between 2 glass plates, covered with butan-1-ol to make a flat gel surface, and allowed to polymerise. The stacking gel was prepared using 2 ml stock acrylamide (22.2% acrylamide, 0.6% bisacrylamide in water), 25 ml gel buffer (pH 6.8), 1.5 ml water, 250 µl ammonium persulphate and 5 µl TEMED. After the separating gel had set, the butan-1-ol was poured off and the stacking gel solution was poured on top. A comb used to form the sample wells was placed into the stacking gel solution and the gel was allowed to polymerise.

Approximately 10-20 µl of the translation mixture from each of the RNA samples was placed in an Eppendorf tube and boiled for 5 min to dissociate the proteins. To each, 60-70 µl sample buffer [0.050 M-

Tris-HCl (pH 6.8), 2% SDS, 5 M urea, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol and 0.5 mg ml^{-1} bromophenol blue] were added. Dalton marker VI was used as the molecular weight marker, and was also boiled for 5 min before adding to the sample well. All protein samples were mixed well and then added to the sample wells. The buffer reservoirs were filled with glycine buffer (0.025 M-Tris-HCl, 0.190 M-glycine, 0.1% SDS). The gel was run for 3-4 h at 3.5 W or until the bromophenol blue band was about 1 cm from the end of the gel.

The gel was removed and stained [2.5 g l^{-1} Coomassie brilliant blue R250, 45% (v/v) methanol, 9% (v/v) acetic acid in water] for approximately 1 h and then destained in 10% methanol, 15% acetic acid in water.

The destained gel was dehydrated in glacial acetic acid for 5 min. Impregnation of the gel with the scintillator PPO, was achieved by adding 100 ml of 20% PPO in glacial acetic acid to the gel for 1½ h. The PPO impregnated gel was placed in distilled water for 1 h or longer, changing the water at least once. This precipitated out the PPO and allowed the gel to be dried down onto a piece of 3 mm paper with a LKB 2003 slab gel dryer. The dried gel was then placed in an autoradiograph film cassette with Kodak OMAT-S film and stored for the required length of time at -70° C. The fluorograph was developed using Kodak D-19 developer (5 min) and fixed using Kodak Unifix (5 min).

Table 3.1

Commonly used buffers and solutions

TE buffer

0.00 M-Tris-HCl (pH 8.0)

0.001 M-EDTA

SSC

0.14 M-NaCl

0.015 M-Na_3 citrate

TBE buffer

0.089 M-Tris-borate

0.089 M-boric acid

0.002 M-EDTA

50 x Denhardt's stock solution

1% ficoll (w/v)

1% PVP

1% BSA

Stored at -20° C

20 x SSPE stock solution

3.6 M-NaCl

0.2 M-Na phosphate

0.02 M-EDTA (pH 8.3)

CHAPTER 4

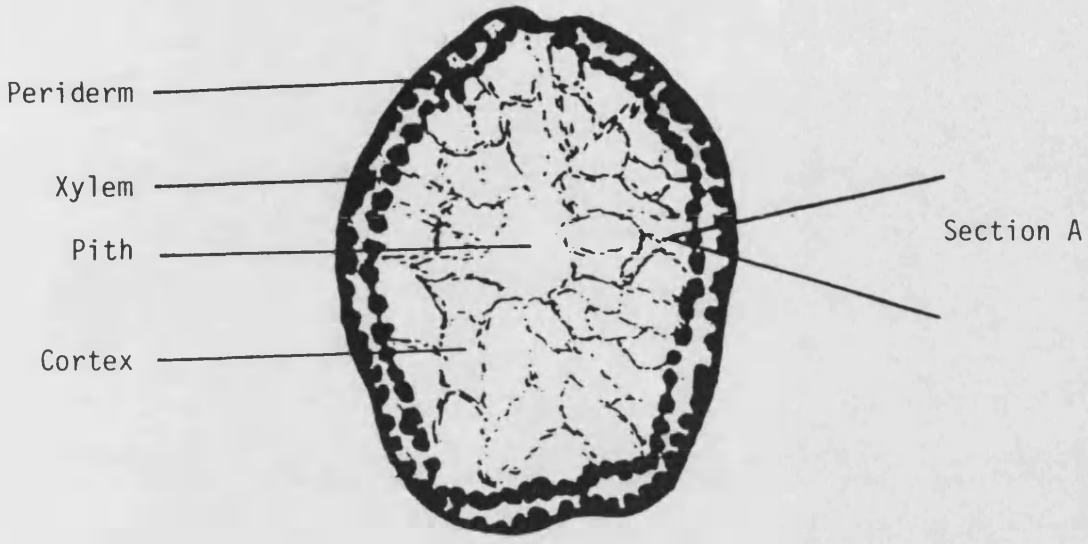
Characterisation of the Potato Tuber as a Developmental System

4.1 Introduction

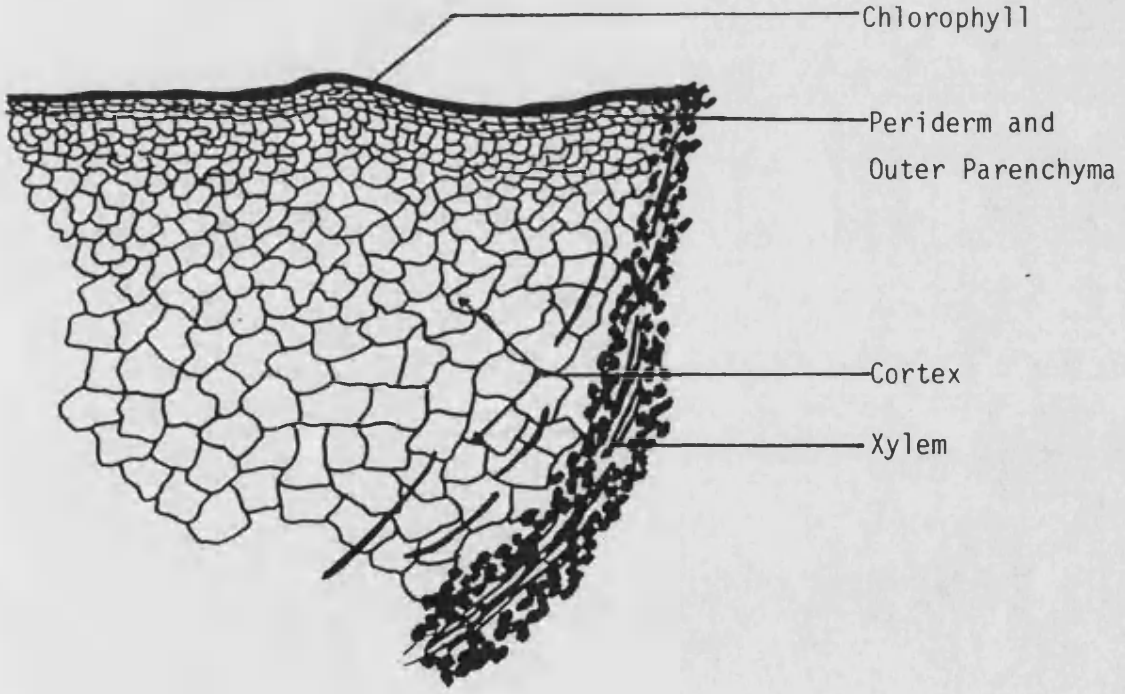
The potato tuber arises from an auxillary bud on the underground stem of the plant, and is therefore simply a shortened stem which has differentiated into a storage organ by expanding radially. The tuber functions in starch synthesis and accumulation, with the final steps of starch synthesis taking place in the amyloplast (Jenner, 1982). There is little information on which of the enzymes involved in starch synthesis are present in the amyloplast (if any). Most of the current information on the metabolism of starch has come from studies on chloroplasts, and it is generally assumed that the mechanisms of starch synthesis are the same in all plastid forms. Work done by Hagemann and Borner (1978) on plastid mutants of *Pelargonium* and *Hordeum* lacking plastid ribosomes, and studies into the genetics of starch synthesis in cereals (Kirk and Tilney-Bassett, 1978) suggest that starch synthesis is controlled by, and the enzymes involved encoded by, nuclear genes.

As with other storage tissues (pea- Millerd and Spencer 1974, and Davies 1976; and watermelon- Newbury *et al.*, 1978), potato tubers can attain high levels of nuclear ploidy. Scott *et al.* (1984a) found potato tuber nuclear ploidy to average approximately 14C for the autotetraploid *Solanum tuberosum*, var. Kennebec. In their study, tissue samples from whole, mature tubers were used. Figures 4.1 and 4.2 show that within the tuber the cells are larger in the pith and cortex regions than in the outer parenchyma and periderm regions. A direct relationship has been found between nuclear DNA ploidy and cell size (Kubitschek, 1974; and Ycas

Figure 4.1 Cross-section of potato tuber showing the location of various cell layers and the location of 'greening' (chlorophyll synthesis).



Cross-section



Enlargement of Section A

Figure 4.2 Tissue samples from A) the outer periderm and parenchyma layers (transverse section), and B) the pith region of potato tuber. Much larger cells are found in the inner pith and cortex regions than in the outer parenchyma and periderm layers of tissue (Zeiss Light Microscope, F10 objective).

A

Scale: 4 cm = 20 μm

B

et al., 1965), so in order to avoid possible variationsin ploidy level associated with variationsin cell size, only the outer parenchyma and periderm tissue layers of the tuber from the autotetraploid plant *Solanum tuberosum*, var. King Edward, were analysed.

This layer of tissue was chosen, not only because it contained cells of relatively uniform size, but also because it is in the outer 3 mm of tuber tissue that 'greening' occurs when tubers are exposed to light (Jadhav and Salunkhe, 1975). Thus, the outer parenchyma and periderm layers of tuber tissue provide an *in vivo* system with which to study the transformation of amyloplasts into functional chloroplasts on exposure to light, as well as the effect light has on other cellular processes.

Nuclear ploidy levels in the cells of the outer parenchyma and periderm layers of tissue were determined by Feulgen cytofluorimetry, which involves the reaction of Feulgen reagent (a solution of fuchsin dye that has been decolourised by sulphur dioxide) to DNA from which the purine bases have been removed by treatment with dilute acid. The exposed aldehyde groups of the sugar deoxyribose combine with the dye to form a magenta-coloured compound, which fluoresces when excited by green light (McLeish and Sunderland, 1961). Leaf tissue, which demonstrates the characteristic tetraploid DNA content, was used as a reference for analysis of tuber tissue ploidy levels over a series of illumination periods.

Determination of chlorophyll content in the outer parenchyma and periderm layers of tuber tissue gave an indication of the extent of amyloplast to chloroplast conversion. Tubers exposed to 0, 4, 8 and 12 days of illumination were used in the following studies, since they

provide a range of illumination periods which include the minimum and maximum chlorophyll synthesis levels. Tubers exposed to 0 days of illumination will be referred to as white tubers, and those exposed to 4, 8 and 12 days of illumination as green tubers.

4.2 Chlorophyll Synthesis in Light Illuminated Potato Tubers

Greening of the periderm and outer parenchyma layers of tissue in potato tubers exposed to illumination was directly visible by eye (Figure 4.3). Using the equation developed by Arnon (1949), based on the work of MacKinney (1941):

$$C = (20.2\ Abs_{645} + 8.02\ Abs_{663}) \times \text{dilution factor},$$

total chlorophyll (chlorophylls a and b) content, C, in non-illuminated and illuminated tissues could be determined from the absorbance values of 80% acetone extracts at 645, 663 nm after correcting for non-specific absorption (Abs_{700}). The total chlorophyll value, C, is expressed in mg chlorophyll per ml, and from this the more commonly used µg chlorophyll per gram fresh weight was determined.

Table 4.1 and Figure 4.4 show an initial lag period followed by an increase in chlorophyll content in tuber tissue with the increase in days of illumination up to approximately 10 days. After 10 days there was no further increase in chlorophyll content and a slight decrease was noticeable by 17 days of illumination, as tissue degradation began to occur. These findings were in excellent agreement with the findings of Anstis and Northcote (1973) and Zhu *et al.* (1984).

4.3 Nuclear Ploidy Analysis of Various Potato Tissues

Samples for cytofluorimetric measurements were taken from leaf, green tuber (4, 8 and 12 days of illumination) and white tuber tissues. However, the large starch granules within the cells of tuber tissue

Figure 4.3 Cross-section of potato tuber after 0 and 12 days of illumination. Chlorophyll accumulation is clearly evident in the outer layers of tissue - comprising the outer parenchyma and periderm layers - of the tuber exposed to 12 days of illumination. Only the outer layers of tissue (2-3 mm) from white and green tubers were used in experiments.

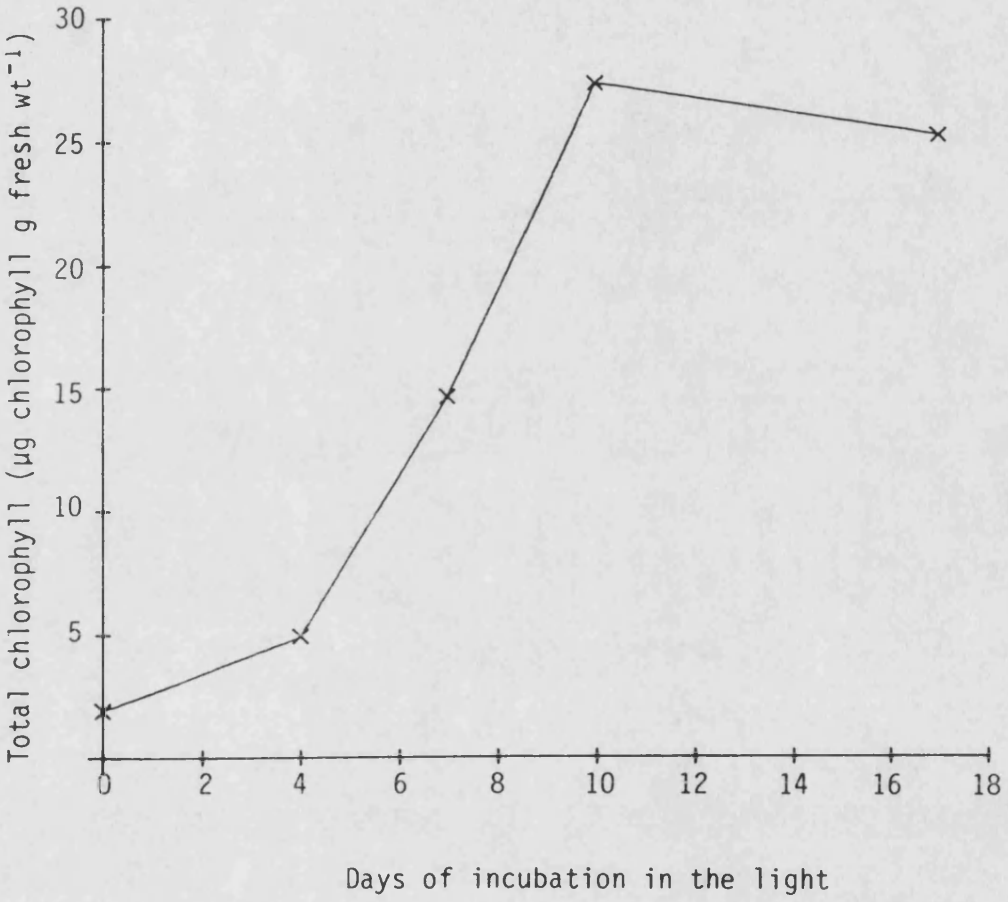
Table 4.1 Using the equation developed by Arnon (1949): $C = (20.2\ Abs_{645} + 8.02\ Abs_{663}) \times$ dilution, total chlorophyll content (C) was determined for potato tubers illuminated for various periods of time

Days of illumination	Abs_{645}	Abs_{663}*	mg chlorophyll ml^{-1}	µg chlorophyll g fresh wt^{-1} †
0	0.015 (2)	0.010 (2)	0.38×10^{-3}	1.9
4	0.030 (2)	0.045 (2)	0.97×10^{-3}	4.9
7	0.075 ± 0.006 (4)	0.175 ± 0.003 (4)	2.92×10^{-3}	14.6
10	0.165 ± 0.008 (4)	0.250 ± 0.004 (4)	5.46×10^{-3}	27.3
17	0.135 ± 0.015 (4)	0.288 ± 0.027 (4)	5.03×10^{-3}	25.2

*mean absorbance value ± SE, with the number of replicate samples in ( )

†See Figure 4.4

Figure 4.4 Changes in the total chlorophyll content (µg chlorophyll g fresh wt^{-1}) of the outer parenchyma and periderm layers of tissue of the potato tuber. Whole tubers were exposed to 14 h illumination per day at an average of 26 µE m^{-2} s^{-1}.



obscured the Feulgen-stained nuclei making accurate cytofluorimetric measurements impossible (Figure 4.5). Treatment of the tissue samples with 1% Macerozyme for 2 h at 32° C was necessary to digest the cell walls partially and allow dispersion of the starch granules, so that nuclei could be isolated and measured.

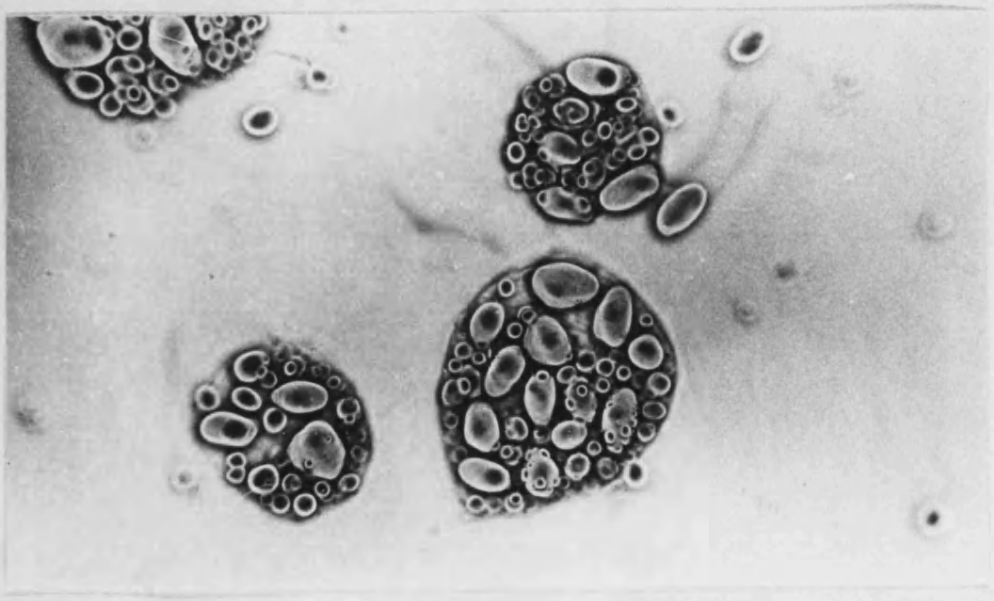
Figure 4.6 shows the frequency distribution of the nuclear DNA levels in King Edward potatoes as measured by cytofluorimetry. The nuclear DNA content in cells of leaf tissue is distributed in one main peak comprising 81% of the nuclei analysed (Table 4.2). This peak is considered to represent the 4X(G1) DNA content and all other C-values were calculated from its median 4C value. Approximately 14% of the leaf nuclei showed 8C (G2) DNA content, 2% 16C DNA content, and 2% 2C DNA content.

From Figure 4.6 and Table 4.2 it is clearly evident that ploidy levels are much higher in tuber tissue than in leaf. White and green (8 and 12 days of illumination) tuber tissue cells show a broad continuum of nuclear DNA content from 4C-64C, with the majority of nuclei in the 16C-32C range. This is also reflected in their average ploidies; 23C, 26C, and 23C respectively for white, green (8 days of illumination), and green (12 days of illumination) tuber tissues. However, green (4 days of illumination) tuber tissue shows a majority of nuclei with DNA contents in the 8C-16C range, with an average ploidy of 15C. This demonstrates a marked decrease in nuclear ploidy, as white tuber tissue is illuminated for up to 4 days.

4.4 Discussion

The transformation of amyloplasts into functional chloroplasts in tuber tissue can be induced by light. However, before the potato tuber

Figure 4.5 Isolated protoplasts from the outer parenchyma layers of tuber tissue showing numerous large starch granules within the cell (Zeiss light microscope, F10 objective) Courtesy of Sarah Jakeman, University of Bath.



Scale: ⊢⊣ = 20 µm
4 cm

Figure 4.6 Frequency distribution of nuclear DNA content in various potato tissues as measured by cytofluorimetry. Markings for ploidy levels were based on the mean tetraploid (4C) absorbance value obtained for leaf tissue nuclei.

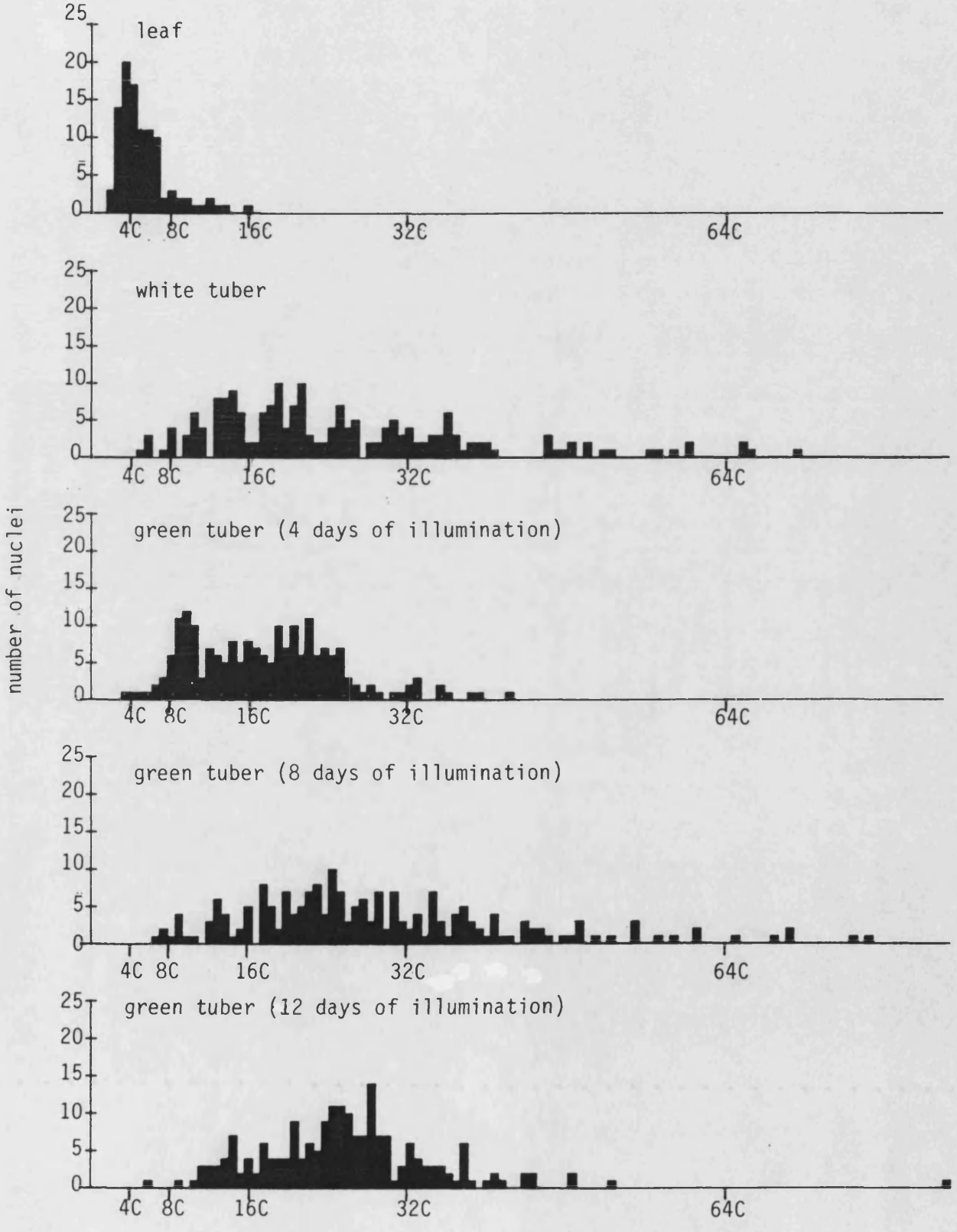


Table 4.2 From Figure 4.6, the percentage of total nuclei with nuclear DNA contents within the following euploid groupings was determined. The groupings were made such that the euploid marker (Figure 4.6) was the median value, with 50% of the values being taken from either side, but not extending into the next grouping.

	Potato Tissue				
Ploidy level	Leaf	White	Green (4)	Green (8)	Green (12)
2C	2%	0%	0%	0%	0%
4C	81%	2%	3%	0.5%	0.5%
8C	14%	13%	29%	9%	6%
16C	2%	44%	57%	39.5%	46%
32C		35%	11%	44%	47%
64C		6%		7%	0.5%
Average	4.8C	23C	15C	26C	23C

can be used as an *in vivo* system with which to analyse this transformation the tissue must be characterised.

A normal whole potato contains very little chlorophyll, but on exposure to light chlorophyll is synthesised in the peripheral layers of tissue (periderm and outer parenchyma). Since the cells in this tissue are fairly uniform in size and contain amyloplasts which are transformed into chloroplasts, functional in chlorophyll synthesis, only this tissue was used in experiments.

Studies on nuclear ploidy can easily be performed by cytofluorimetric measurements using Feulgen reagent. A step involving Macerozyme pre-treatment of the tissue was added to the tissue preparation protocol and resulted in preparations yielding well isolated nuclei unobstructed by large starch granules, and decreased unspecific staining.

All nuclei measured were grouped into C-values based on the 4C (tetraploid) peak of leaf tissue nuclei. These groupings are not intended to suggest that all nuclei possess only euploid DNA content. Rather, with the high ploidy levels shown in tuber tissue aneuploid nuclei are bound to exist, due to mitotic irregularities in synthesis and division of such large quantities of DNA. Unfortunately, the above procedure was not sensitive enough to detect aneuploid nuclei from euploid nuclei. Therefore, the euploid groupings are arbitrary groupings used only to give an idea of ploidy level trends.

From Figure 4.6 and Table 4.2 there is no significant difference in the nuclear ploidy of white and green (8 and 12 days of illumination) tuber tissue where the average ploidy is 23C, 26C and 23C respectively. However, with the onset of greening, white to green (4 days of illumination) tuber tissue, there is a decrease in average ploidy level (23C to

15C, respectively). One possible explanation for this decrease in ploidy level with greening may be the initiation of synchronised cellular division in tuber tissue on exposure to light.

In the cells of pea cotyledon mitosis is inhibited at an early developmental stage (Davies, 1976). From this and based on the constant ratio of DNA to cell volume found in a variety of prokaryotic and eukaryotic cells (Kubitschek, 1974; Yeas *et al.*, 1965), Davies suggested that cells in the cotyledons of pea ceased cell division but continued to grow and increase in cell volume. This increase in cell volume triggered off repeated rounds of DNA synthesis until no further increase in cell volume could occur. A similar mechanism could be operating in tuber tissue. On exposure to light, synchronised cellular division could be triggered (Harris, 1978), thus explaining the decreased ploidy level found in green (4 days of illumination) tuber tissue. The further increase in ploidy level with illumination time may be due to cell expansion with further DNA synthesis and inhibition of cell division or very slow progression of the cells through the cell cycle. Jenner (1982) found that the cells of tuber tissue retain their capacity for cell division throughout their growth. However, the rate at which cell division occurs and its regulation are probably due to a number of factors. Two controlling factors have been proposed: (1) assimilate supply, and (2) phytohormones (Lovell and Booth, 1967; Moorby, 1978; Mares and Marschner, 1980). Further study into the greening event in tuber tissue is needed to determine the regulation of cell division.

There are several proposals for the high ploidy levels found in storage tissues; one being the above proposal by Davies (1976) of inhibition of cell division. Alternatively, Scharpé and van Parijs (1973)

suggested that polyploidisation in the pea cotyledon system is directly related to storage protein synthesis by virtue of a gene dosage effect; *i.e.*, it is a means of making available extra copies of those cistrons involved in synthesis of storage proteins. However, Millard and Spencer (1974) found that synthesis of extra DNA does not result in a proportional increase in RNA synthesis by the developing pea cotyledon cells. Madison *et al.* (1976) found that extra DNA does not seem to be obligatory for the synthesis of reserve proteins in cereals, nor is the final protein level in legumes closely linked with DNA content. In contrast, Nagl (1973) reported increases in RNA synthesis in polyploid cells over similar diploid cells in *Allium carinatum* roots.

The role of extra nuclear DNA in potato tuber tissue is not known, nor is the specific mechanism by which polyploidisation occurs and is controlled. However, these studies do provide useful information on the tissue which was chosen for study, even if they do ask more questions than they answer.

CHAPTER 5

Qualitative Analysis of Potato Plastid DNA

5.1 Introduction

The previous chapter characterised the potato tuber as a developmental organ which exhibits a variety of changes when exposed to light. This chapter characterises the pt DNA of potato, using the ct DNA of potato leaf as a representative of the DNA of all plastid forms in potato.

The fragment patterns produced by restriction enzyme digestions of potato ct DNA allowed for comparison with the published patterns of Schiller *et al.* (1982), whose work was on the dihaploid potato HH258. From this comparison and based on the extensively mapped plastome of tobacco (Sugita *et al.*, 1984; Hildebrand *et al.*, 1985; Seyer *et al.*, 1981; and Fluhr *et al.*, 1983), also of the family *Solanaceae*, a restriction endonuclease cleavage site map of potato has been proposed, see Discussion, 5.5.

5.2 Restriction Endonuclease Digestion Analysis of Potato Chloroplast DNA

5.2.1 Chloroplast DNA purification

Plastid DNA was purified from isolated potato leaf chloroplasts. This was due to the ease of isolation compared with tuber amyloplasts, where the large starch granules act as disruptive agents. However, the extraction of intact ct DNA is made difficult by the fact that: (1) it is a relatively large molecule (156 kbp for potato), (2) it constitutes only a minor fraction of the total cellular DNA, and (3) its density is similar to nuclear DNA. Intact ct DNA can be prepared following the procedure described in Section 3.4, where chloroplasts are first isolated and then ct DNA is extracted from them.

The difference in buoyant density of ct DNA and nuclear DNA in an isopynic CsCl/ethidium bromide density gradient is then utilised to separate the ct DNA from any contaminating nuclear DNA. The resulting ct DNA is of a level of purity adequate for restriction endonuclease digestion analysis.

The preparation of whole intact chloroplasts is a necessary preliminary step before circular ct DNA can be isolated. Isopynic sucrose gradient centrifugation was used to isolate intact chloroplasts. Since chloroplasts are practically impermeable to sucrose, the organelle contracts in the hypertonic solution and there is a large scale loss of its soluble constituents. This has no adverse effect on the ct DNA quality and purity, since the organelle retains its compact form and the ct DNA, which is generally membrane-based, remains within the organelle (Herrmann, 1982). Morphological analysis *via* light microscopy of a sample of the chloroplast band from a sucrose gradient reveals a majority of intact chloroplasts, Figure 5.1. The unbroken chloroplasts appear refractive, encircled by a 'halo', while fragmented chloroplasts appear dark with no 'halo'.

Since most ct DNA is attached to membranes, lysis is a very critical step in the isolation of circular DNA from chloroplasts. Sodium dodecylsarcosinate (5%) was used to lyse the chloroplasts and complete lysis was evident when only very tiny thylakoid remnants were visible by light microscopy (Figure 5.1).

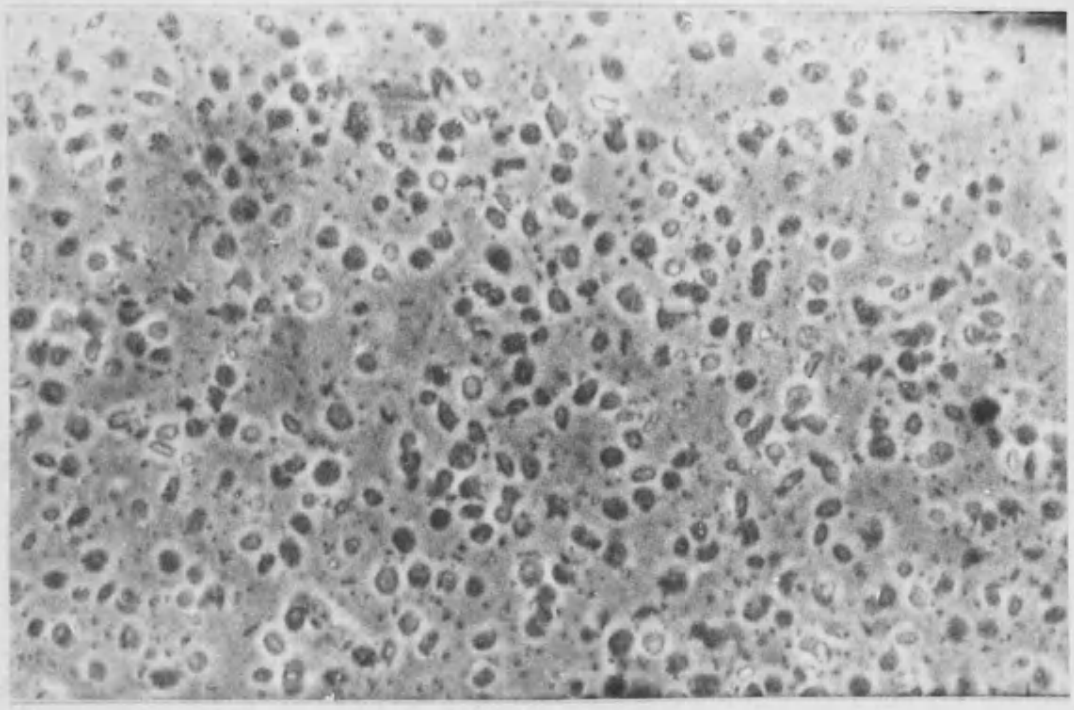
Due to the large amounts of protein remaining after lysis, phenol/$CHCl_3$ steps were added to deproteinise the DNA sample. CsCl/ethidium bromide density gradient centrifugation of the ct DNA sample typically revealed two bands (Figure 5.2). The upper band contains nicked, linear ct DNA and any nuclear contamination and the lower band contains intact

Figure 5.1 Chloroplasts before and after lysis:

A Chloroplasts extracted from a 20-55% sucrose density gradient spun at 80,000 g for 35 min. Intact chloroplasts, those encircled by halos, are clearly evident.

B Only thylakoid remnants are present after lysis of chloroplasts by treatment with 5% sodium sarcosinate.

A



Scale: $\frac{\text{⊢}}{4\text{ cm}} = 5\ \mu m$

B

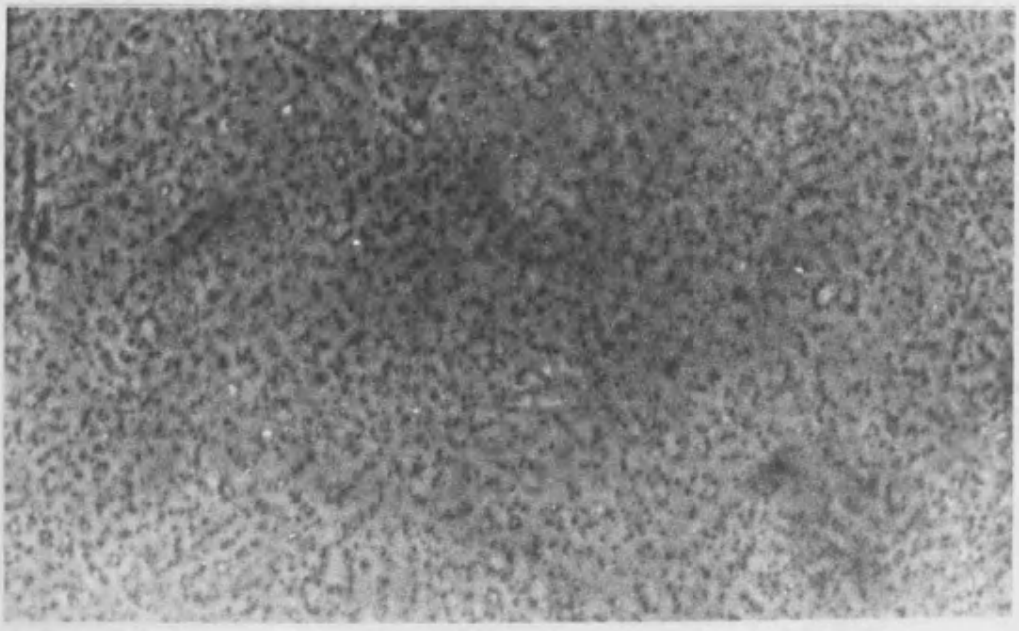
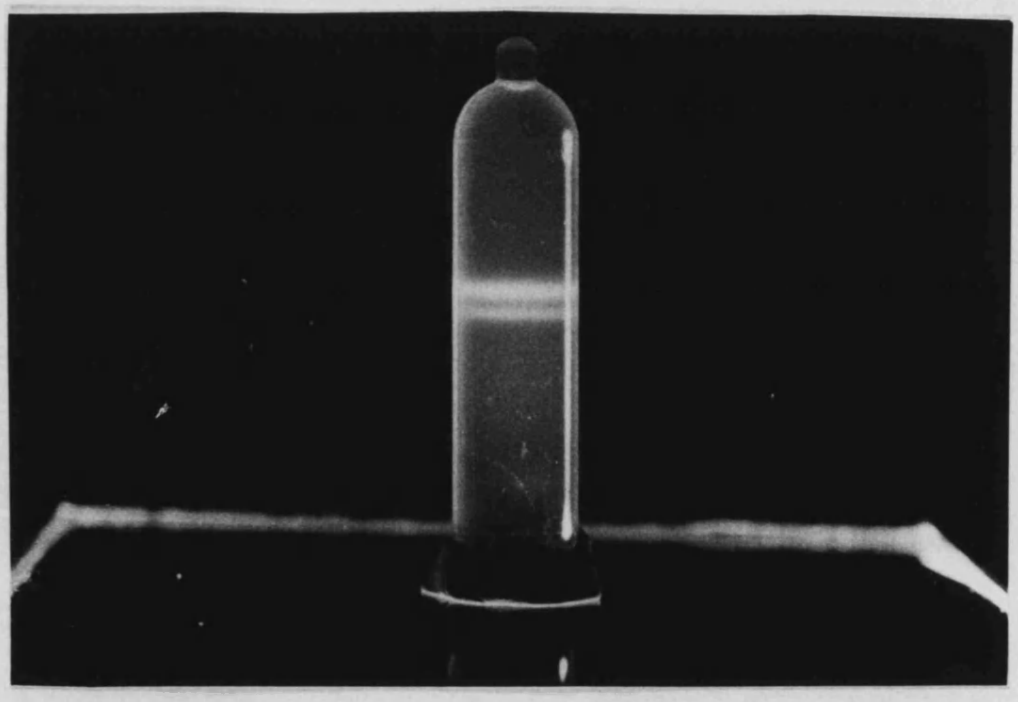


Figure 5.2 Closed circular ct DNA isolation.
Separation of linear and nicked ct DNA, and contaminating nuclear DNA (top band) from closed circular ct DNA (bottom band) by centrifugation in a CsCl/ethidium bromide equilibrium gradient. RNA is pelleted against the side of the tube, in a narrow vertical line.



circular DNA (Herrmann, 1982). The lower band was always taken and used as the purified ct DNA preparation.

5.2.2 Fragment pattern analysis

In order to show the nature of extracted ct DNA from potato, its purity, intactness and similarities to other closely related plant pt DNAs, restriction endonuclease digestions were performed and the fragment patterns were analysed by agarose gel electrophoresis. In my experiments, potato leaf ct DNA was digested with 3 hexanucleotide-specific restriction endonucleases; Sal I, Pst I and kpn I. The digest fragments from a double-digest of λ DNA using restriction enzymes Bam HI and Eco RI, and from a Pst I single-digest of λ DNA were used as molecular weight markers. The cleaved ct DNA and the λ standards were run on 0.7% agarose gels, which give an efficient range of separation of linear molecules from approximately 0.8 - 10 kbp (Maniatis *et al.*, 1982). Figure 5.3 shows ct DNA digestion fragments produced by Sal I and kpn I, with the Bam HI/ Eco RI double-digest of λ DNA as a marker. Figure 5.4 shows Sal I and Pst I digestion fragments of ct DNA, using a Pst I digest of λ DNA as a marker. The Pst I λ DNA marker showed an additional band, which is most likely due to reannealing of cohesive ends.

The size of the restricted potato ct DNA fragments can be estimated from their electrophoretic mobility by comparison with those of the DNA markers. A plot of mobility *versus* DNA fragment size of the λ DNA markers results in a smooth curve (Figure 5.5) from which the ct DNA fragment sizes were estimated. Table 5.1 shows a comparison of the restriction enzyme fragment size estimates obtained from Sal I and kpn I digests, and those obtained by Schiller *et al.* (1982), who used the same restriction enzymes but with ct DNA from a dihaploid potato stock [HH258 (2n = 24)]. Accurate size estimates of the larger fragments were

Figure 5.3 Restriction endonuclease digestion fragments produced by the digestion of purified potato ct DNA with Sal I (track 1) and Kpn I (tracks 2 and 3) and separated by electrophoresis on a 0.7% agarose gel. Phage λ DNA digested in a double-digest with Bam HI and Eco RI was used as a marker, units are in kbp. Track 1 shows the presence of either some undigested ct DNA or contaminating nuclear DNA at the origin. The background smear in all tracks is most likely due to nuclear DNA contamination.

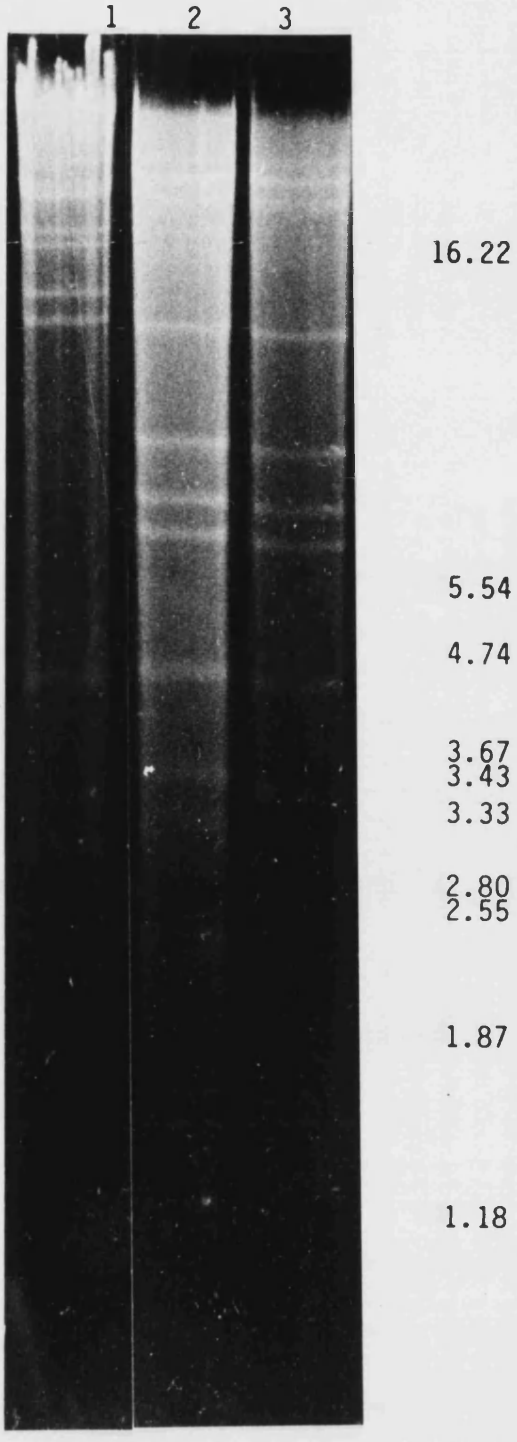
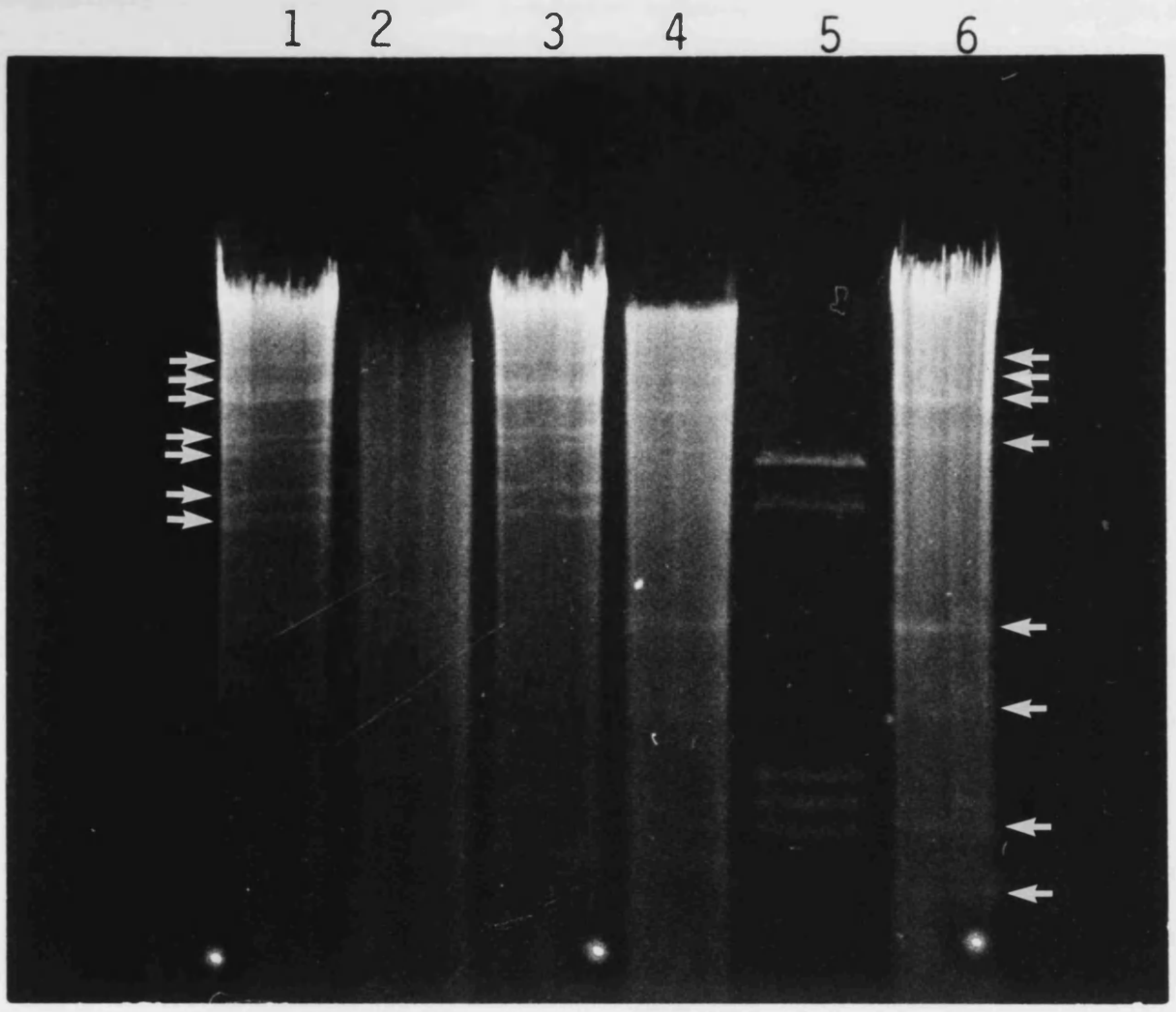

1
2
3
16.22
5.54
4.74
3.67
3.43
3.33
2.80
2.55
1.87
1.18

Figure 5.4 Restriction endonuclease digestion fragments produced by the digestion of purified potato ct DNA with Sal I (tracks 1 and 3) and Pst I (tracks 4 and 6). Track 2 is phage λ DNA digested with Sal I, however not enough DNA was applied to the gel. Track 5 is phage λ DNA digested with Pst I, the sizes of the fragments are given in kbp. The additional band in the Pst I digest of DNA is probably due to reannealed 11.5 kbp fragments.

The Sal I restriction endonuclease fragments of ct DNA (tracks 1 and 3) are marked by arrows. Only the higher mol wt fragments were detectable. These are similar to those found by Schiller *et al.* (1982), 27.4, 24.8, 21.5, 17.1, 15.9, 12.6, 11.4, 4.1, 2.7 kbp. The fragments produced from the ct DNA digest with Pst I (tracks 4 and 6) are very faint; however, in the original photograph the banding patterns produced (marked by arrows) are very similar to those obtained by Schiller *et al.* (1982), who obtained fragments of 25.0, 20.8, 18.9, 18.4, 15.0, 7.9, 5.95, 4.55, 2.8 and 2.5 kbp.



11.5
additional band

5.25
4.80
4.56

Figure 5.5 Standard curve showing the relative electrophoretic mobilities of the products of an Eco RI-Bam HI double-digest of phage λ DNA run on a 0.7% agarose slab gel. This curve was used to estimate the ct DNA fragment sizes.

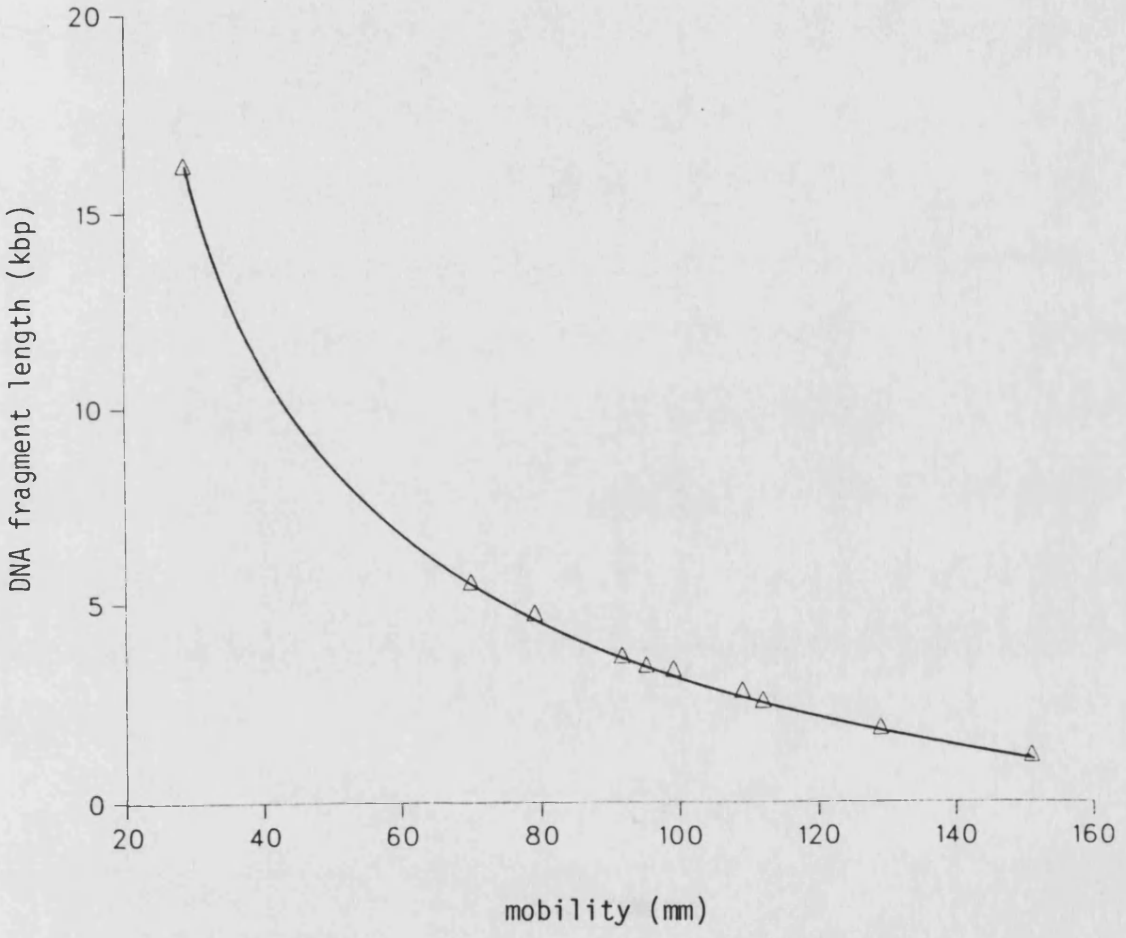


Table 5.1 Size estimations of the fragments of potato ct DNA produced by digestion with restriction endonucleases Sal I and Kpn I in comparison with those found by Schiller *et al.* using the same enzymes, but with the dihaploid potato HH 258.

Sal I		Kpn I	
kbp[a]	kbp[b]	kbp[a]	kbp[b]
27.4		33.8	
24.8		26.6 (2x)	
21.5		22.3	
17.1		12.8	10.5
15.9	16.0	8.4	7.5
12.6	12.5	7.0	6.6
11.4	11.0	6.6	6.0
4.1	4.5	4.25	4.4
2.7	3.0	3.4	3.5
		3.0	3.0
		0.82 (2x)	ND
		0.71	ND
		0.54	ND

[a] From Schiller *et al.* (1982)

[b] From Figure 5.3 and 5.5

(ND = not detectable)

not possible, due to the limitations of the efficient separation range of the gel and the marker DNA not extending into the higher molecular weight range. However, the fragments estimated were in very close agreement with those found by Schiller *et al.* (1982) and the larger fragments, which could not be accurately measured, gave similar digest patterns to those found by Schiller *et al.* (1982).

Nuclear DNA contamination is evident in almost all ct DNA digests as a broad background streak or fluorescence at the origin. However, Pst I does not cleave nuclear DNA because of methylation sensitivity (Bowman and Dyer, 1982), and therefore the nuclear DNA should remain near the origin. However, from Figure 5.4 it appears that some cleavage of nuclear DNA by Pst I may have occurred or the background streak represents nuclear or ct DNA degradation. The strong fluorescence at the origin of most tracks could also be undigested ct DNA.

5.3 Discussion

It is generally assumed that within one species the pt DNA is the same in all the plastid forms. The DNA from *Narcissus* chromoplasts has been found to be the same length as that found in the chloroplasts (Falk *et al.*, 1974) and restriction endonuclease digestions have shown that the chromoplast and chloroplast DNAs generate the same fragments (Thomson, 1980). In *Panicum maximum*, both the mesophyll and bundle-sheath cells contain the same pt DNA (Walbot, 1977). Reassociation rate kinetic analysis indicate that root pt DNA and ct DNA are essentially the same in spinach (Scott and Possingham, 1980) and beet (Tymns *et al.*, 1983). Scott *et al.* (1984a) demonstrated by restriction enzyme analysis and re-association kinetics that chloroplast and amyloplast DNA is essentially the same in the potato *Solanum tuberosum* L. var. Kennebec. In this study,

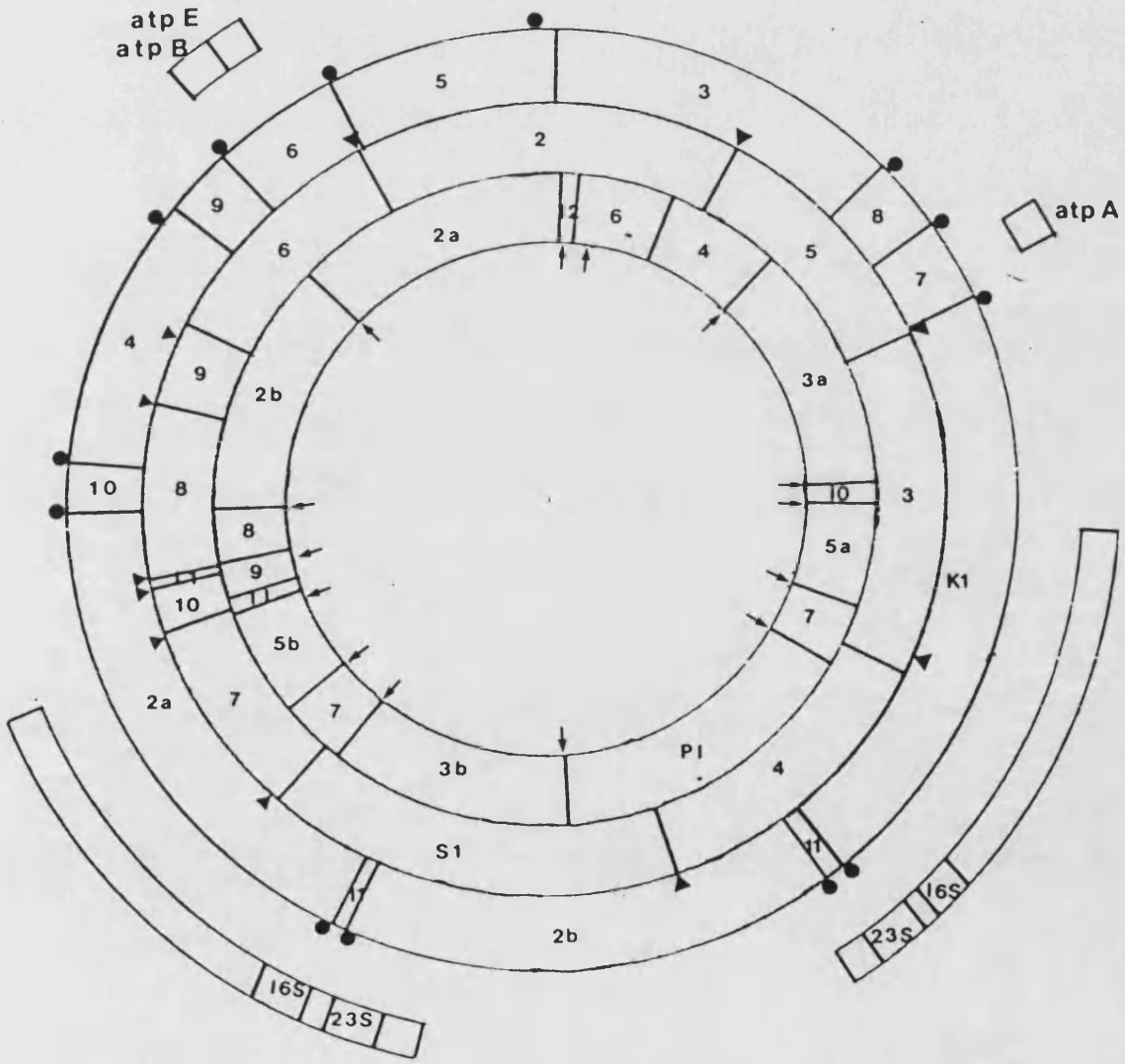
restriction enzyme analysis was used to determine ct DNA purity and intactness after extraction, as well as to demonstrate the intra-familial similarity amongst pt DNAs.

Identical restriction enzyme patterns of pt DNA were found when the potato variety King Edward was compared in the dihaploid potato, HH258, used by Schiller *et al.* (1982). Within the family *Solanaceae* (to which potato belongs), Fluhr and Edelman (1981) found site changes sufficiently infrequent between the genera *Atropa, Nicotiana,* and *Petunia,* to allow comparisons of restriction enzyme patterns for mapping differences among genera.

In this study, the well-documented restriction enzyme patterns of tobacco pt DNA (*Nicotiana tabacum*) were compared to the digest patterns resulting from the cleavage of potato pt DNA with the same enzymes, using the potato pt DNA fragment sizes determined by Schiller *et al.* (1982) (Table 5.1). The tobacco and potato pt DNAs are of approximately the same size, 160 kbp and 156 kbp respectively. The digest patterns for potato pt DNA were found to be very similar to those for tobacco pt DNA, and from the differences in fragment size a cleavage site map for potato is proposed (Figure 5.6), based on the cleavage site maps of tobacco.

The tobacco pt DNA is organised into four distinct regions; inverted repeat regions are separated by a small and a large single-copy region. As found in other plastomes (Tassopula and Kung, 1984), the small single-copy region in tobacco and potato pt DNAs appears to be highly conserved with no apparent variations in restriction sites or size. Most deviations from the tobacco map are located in the large single-copy region. This seems to be generally true for plastomes from many other plants (Herrmann *et al.*, 1980). A recently published endonuclease cleavage site map of

Figure 5.6 The physical map of *Nicotiana tabacum* ct DNA with the proposed cleavage sites for potato ct DNA. The concentric circles represent *N. tabacum* cleaved with Kpn I (outer circle; Hildebrand *et al.*, 1985), Sal I (middle circle; Seyer *et al.*, 1985), and Pst 1 (inner circle; Cannon *et al.*, 1985). The ● , ▲ , † represent the proposed cleavage sites of potato ct DNA with the respective enzymes Kpn I, Sal I and Pst I. Tobacco and potato ct DNAs yield very similar endonuclease digestion fragments, therefore the cleavage site map proposed for potato is very similar to that found for tobacco. The sites for the inserted repeats, containing the 16S and 23S rRNA genes, and the gene sequences for the α, β and ε subunits of CF_1 of ATP synthase are shown.


atp E
atp B
atp A
5
3
2
6
9
6
2a
12
6
4
5
8
7
4
9
2b
3a
10
8
3
10
8
5a
9
K1
10
7
5b
2a
7
7
3b
P1
4
S1
11
11
2b
16S
23S
16S
23S

tomato pt DNA (Phillips, 1985a,b), also shows digest fragment patterns similar to potato with no apparent variations in the small single-copy region and most occurring in the large single-copy region. The proposed map of potato pt DNA requires verification by: (1) double and partial digestion analysis, to determine adjacent fragments, and (2) hybridisation studies of the restriction fragments with known gene probes, to determine the location of specific genes.

CHAPTER 6

Quantification of Plastid DNA

6.1 Introduction

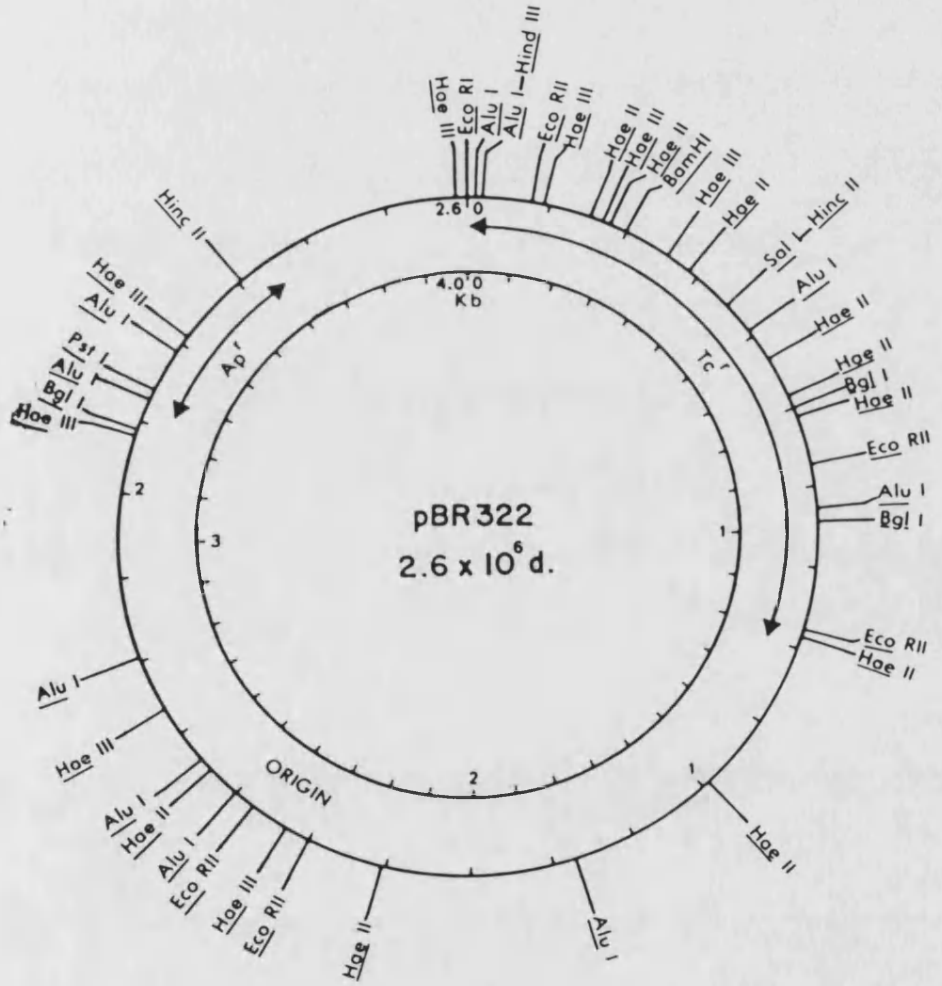
The results from nuclear ploidy analysis of various potato tissues, as presented in Chapter 4, show that higher ploidy levels are found in tuber tissue than in leaf tissue. This chapter investigates the possible parallel correlation between nuclear ploidy and pt DNA levels in potato tissues, and also examines the effect greening has on pt DNA levels in tuber tissue.

The potato tuber functions in starch synthesis and storage with the final steps of starch synthesis and deposition taking place in the amyloplast (Figure 1.4). The mechanism of starch synthesis in virtually all plastids, including amyloplasts, chloroplasts and other plastid types, is believed to be similar (Scott *et al.*, 1984a). Studies of starch synthesis in pt DNA mutants of *Pelargonium* and barley, which lack plastid ribosomes (Hagemann and Borner, 1978) and the genetics of starch accumulation in cereals (Kirk and Tilney-Bassett, 1978, indicate that starch synthesis is regulated by nuclear genes and the enzymes involved are encoded on the nuclear genome. Hagemann and Borner (1978) and Scott *et al.* (1982b) also found that in the albino plastids of the barley mutant 'albostrians' the synthesis of pt DNA and the maintenance of the albino plastids continued in the absence of expression of the pt DNA. This led Scott *et al.* (1984a) to propose that the starch-accumulating function of potato amyloplasts might not involve transcription of pt DNA and that the levels of pt DNA in potato amyloplasts might be relatively low. Although Scott *et al.* (1984a)

found fewer plastids per cell in tuber tissue (approximately 40) than in leaf tissue (approximately 135), the number of plastome copies per plastid in tuber tissue was 195, compared to 22 plastome copies per plastid found in leaf tissue. Using reassociation rate kinetics, they calculated the levels of pt DNA in the total DNA population and found: 5.2% pt DNA in tuber tissue, 7.6% pt DNA in leaf, 3.4% pt DNA in petioles, 3.0% pt DNA in stems, and 1.0% pt DNA in roots. These studies demonstrated that high levels of pt DNA ploidy were attained in mature potato tubers.

My preliminary studies into the pt DNA levels in leaf and tuber tissue were already begun when Scott *et al.* (1984a) published their findings. Results obtained from reassociation kinetics using radio-labelled ct DNA as a probe differed from those found by Scott *et al.* (1984a), giving 4% pt DNA in leaf tissue and 12% pt DNA in tuber tissue. This discrepancy led to the use of dot hybridisation analysis as an alternative way of detecting pt DNA levels in total DNA populations. Using this technique, leaf tissue and tuber tissues, exposed to 0, 4, and 8 days of illumination, were analysed. The dots of total DNA were probed with wheat ct DNA fragments cloned into *p*BR322 DNA (Figure 6.1), thus avoiding the possibility of nuclear DNA contamination of the probe - a problem faced when using ct DNA as a probe. Two plasmid probes were used, one containing the sixth Pst I fragment (P6) of wheat ct DNA and the other containing the second Bam HI fragment (B2) of wheat ct DNA. The P6 fragment contains the genes for the 16S rRNA and part of the 23S rRNA. The B2 fragment contains the genes for the large subunit of RuBPcarboxylase (rbcL), the β and ε subunits of CF_1 of ATP synthase (atpB, atpE) and part of the cytochrome f gene

Figure 6.1 The circular restriction map of *p*BR322. The sites of Ap^r and Tc^r are shown, as well as the position of restriction endonuclease cleavage (From Bolivar *et al.*, 1977).



(petA) (Figure 6.2). This method allows for a quick assessment and quantification of pt DNA levels in the various potato tissues.

6.2 Quantification of Plastid DNA Levels in Various Potato Tissues by Reassociation Rate Kinetics

6.2.1 Radiolabelled ct DNA probe preparation

The radiolabelled DNA used as the hybridisation probe for kinetic measurements was ^{3}H-TTP labelled ct DNA. The ct DNA was labelled *in vitro* by nick-translation to a specific activity of approximately 1×10^6 cpm μg^{-1} DNA, with 10-20% incorporation of the radiolabel into DNA. This provided an adequate amount of radioactivity with which to detect hybridisation using S_1 nuclease criteria.

The radiolabelled DNA was fractionated from the residual nucleotides by elution of the nick-translation reaction mixture through a Sephadex G-50 column using TE buffer. Figure 6.3 shows the typical elution profile of DNA and nucleotides as detected by scintillation counting. The DNA fractions were pooled and used as the radiolabelled probe in reassociation rate kinetic studies.

6.2.2 Kinetics of reassociation

To determine the fraction of total cellular DNA represented by pt DNA, total DNA driven hybridisations to ^{3}H-TTP labelled ct DNA probe were performed. The kinetics of reassociation of probe to the fraction of pt DNA in the total DNA population were determined, and from this the percentage pt DNA was calculated.

The rate of reassociation is second order

$$dC/dt = K(C)^2, \qquad (1)$$

where C is the concentration of single-stranded DNA, K is the second order rate constant and t is time. This study is concerned only with

Figure 6.2 Restriction endonuclease map of wheat chloroplast DNA showing the recognition sites for Pst I and Sal GI and the position of the 9.6 kbp Bam HI (B_2) fragment (Bowman *et al.*, 1981). The map also shows the positions and directions of transcription of the genes for cytochrome f (petA), the large subunit of RuBPcarboxylase (rbcL) and the α, β and ε subunits of ATP synthase (Howe *et al.*, 1982a, b; 1983). Also shown are the positions and directions of transcription of the ribosomal RNA genes within the inverted repeat sequence (Bowman *et al.*, 1981). (From Willey *et al.*, 1984).

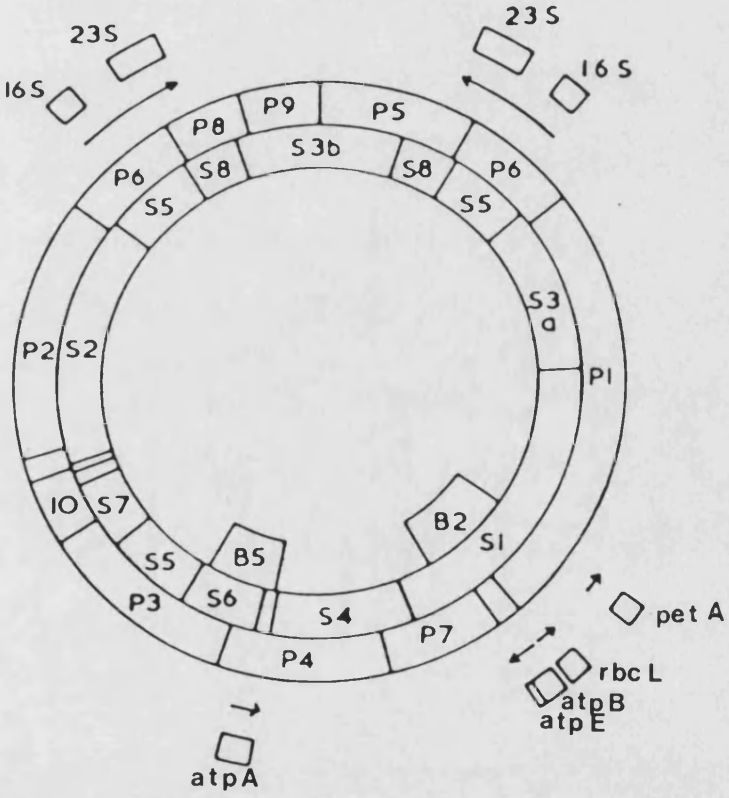
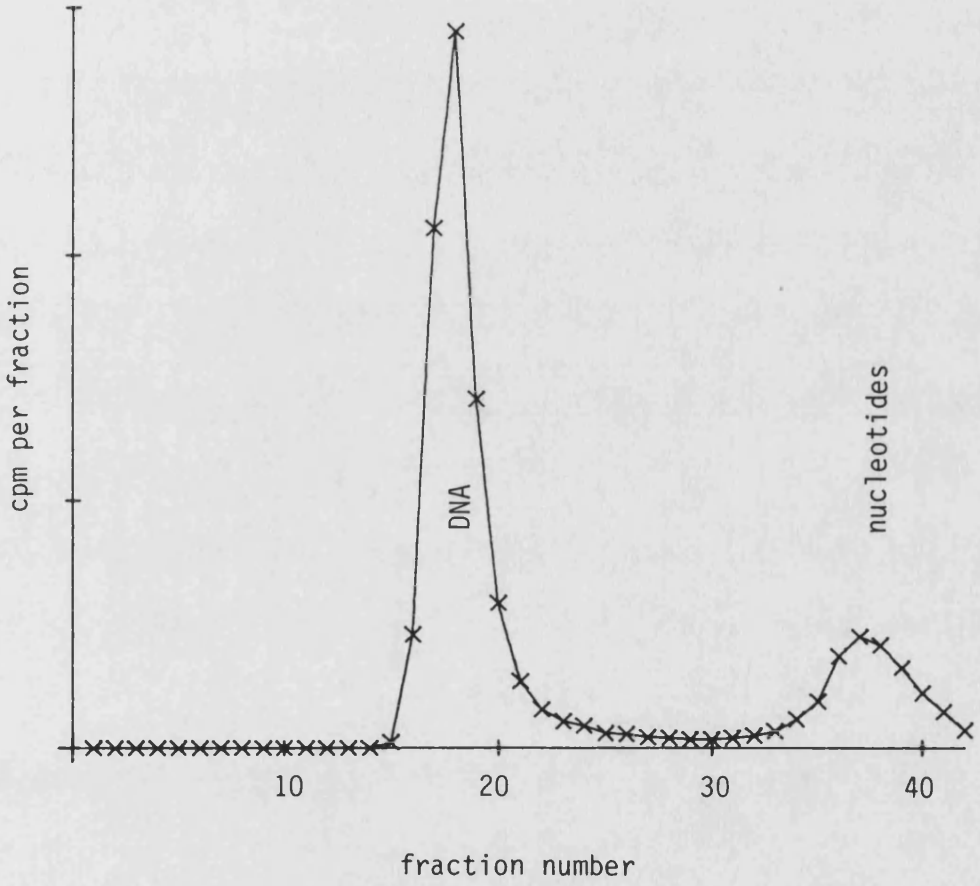

23S
16S
23S
16S
P8
P9
P5
S3b
S8
S8
P6
P6
S5
S5
S3
a
P2
S2
P1
10
S7
B2
S1
S5
B5
P3
S6
S4
P7
P4
pet A
rbc L
atpB
atpE
atpA

Figure 6.3 Typical elution profile of radiolabelled DNA and residual radiolabelled nucleotides through a Sephadex G-50 column as determined by scintillation counting. All nick-translation reactions of ct, *p*BR322-B_2, *p*BR322-P_6, M13-α,β or M13-c DNAs resulted in a similar profile, though with varying amounts of radiolabel incorporation into DNA.



the concentration of probe and therefore the concentration factor C is designated P following the method of Chilton *et al.* (1974). When equation (1) is integrated between P_0, the initial single-stranded concentration in moles of nucleotides l^{-1} and P during the period of t = 0 to t, in s, the following equation is obtained:

$$P/P_0 = 1/(1 + KP_0t), \qquad (2)$$

where P/P_0 is the function of DNA remaining single-stranded at time t. The amount of reassociation is therefore a function of the product P_0t. This has led to the standard representation of reassociation kinetic data, the C_0t curve (or P_0t in this work), where C/C_0 is plotted *versus* C_0t (Britten and Kohne, 1968; Chelm, 1982).

6.2.3 Determination of the percentage pt DNA in total DNA samples

After the reassociation reaction is carried out, each time point must be analysed to determine what fraction of the probe is in duplex form. The two most common procedures used to assay for duplex formation are the separation of double- and single-stranded DNA by hydroxylapatite chromatography or the resistance of double-stranded DNA to digestion by S1-nuclease. In this study only the S1-nuclease assay was used.

Equation (3) is used to give the percentage single-stranded or P/P_0:

$$P/P_0 \text{ (\% single-stranded)} = 1 - \frac{(S_1 \text{ cpm } P_0t = X) - (S_1 \text{ cpm } P_0t = 0)}{(\text{total cpm}) - (S_1 \text{ cpm } P_0t = 0)} \qquad (3)$$

where S_I-nuclease resistant cpm at $P_0t = X$ minus the S_1-nuclease resistant cpm at $P_0t = 0$ is divided by the total cpm in a similar reaction aliquot minus the S_1-nuclease resistant cpm at $P_0t = 0$. The fraction double-

stranded is then subtracted from 1 to give the fraction remaining single-stranded. The percentage single-stranded can be plotted *versus* P_0t to yield a P_0t curve.

Alternatively, the logistic equation can be used for a better fit of the data:

$$Y = A + C/\{1 + Exp\ [- B(x-M)]\} \qquad (4)$$

where Y is P/P_0, x is $\log_e P_0t$, and A, B, C and M are parameters. This equation is equivilant to equation (2) where A is 0, C is 1 and B is -1. Instead of restricting these parameters (only A is restricted to 0) they are allowed to free-float (Gupta *et al.*, 1981) so as to minimise the error. When tested using a 95% confidence interval, C was not significantly different from 1. The P_0t curves obtained from logistics equation are shown in Figure 6.4. The leftward shift in the curves indicates an increase in the percentage pt DNA in the total DNA sample population.

Alternatively, the rearranged form of equation (2):

$$1/P/P_0 = 1 + kP_0t \qquad (5)$$

which represents the equation for a straight line, can be used to plot data (Figure 6.5). Linear regression analysis of the data, in the form of P_0t (X-values) and $1/P/P_0$ (Y-values), gives the least squares standard deviation fit line through the data points. Linear regression analysis determines the rate, k, of the best fit line and this value is related to $P_0t_{\frac{1}{2}}$, which indicates the point (P_0t) when 50% single-stranded DNA remains. The percentage pt DNA in a total DNA sample can be determined using the equation:

$$[(P_0t_{\frac{1}{2}}\ \text{probe}/P_0t_{\frac{1}{2}}\ \text{total DNA}) \times 0.5\ \mu g] - 0.5\ \mu g/\text{total DNA}\ \mu g \qquad (6)$$

where the $P_0t_{\frac{1}{2}}$ ratio of probe to total DNA is multiplied by the amount

Figure 6.4 Reassociation kinetics of radiobalelled potato ct DNA (probe) in the presence of total potato DNA. Aliquots were taken at various time intervals during the reassociation reaction from leaf (X) and tuber (□) tissues. S1-nuclease criteria were used to determine the percentage of the probe remaining single-stranded. The reassociation of potato ct DNA to itself is also shown (△). Curves were fit using the logistics equation (see text) and the actual data points are shown. Po = concentration of probe DNA, t = elapsed time of measurement.

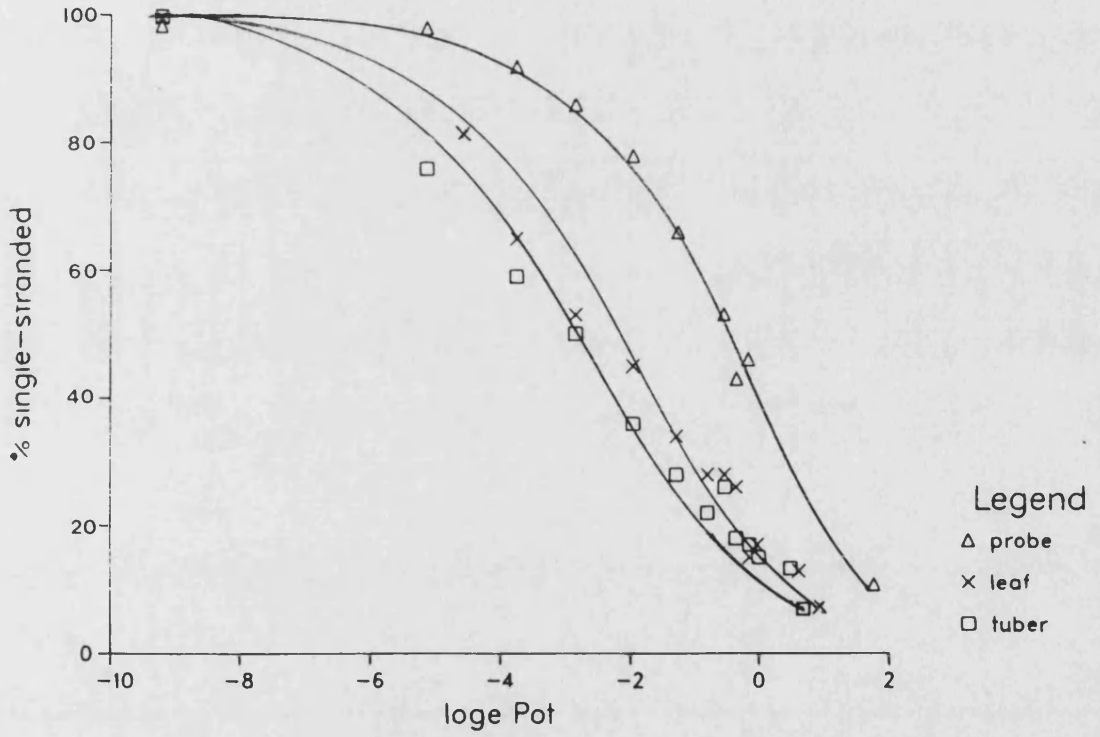
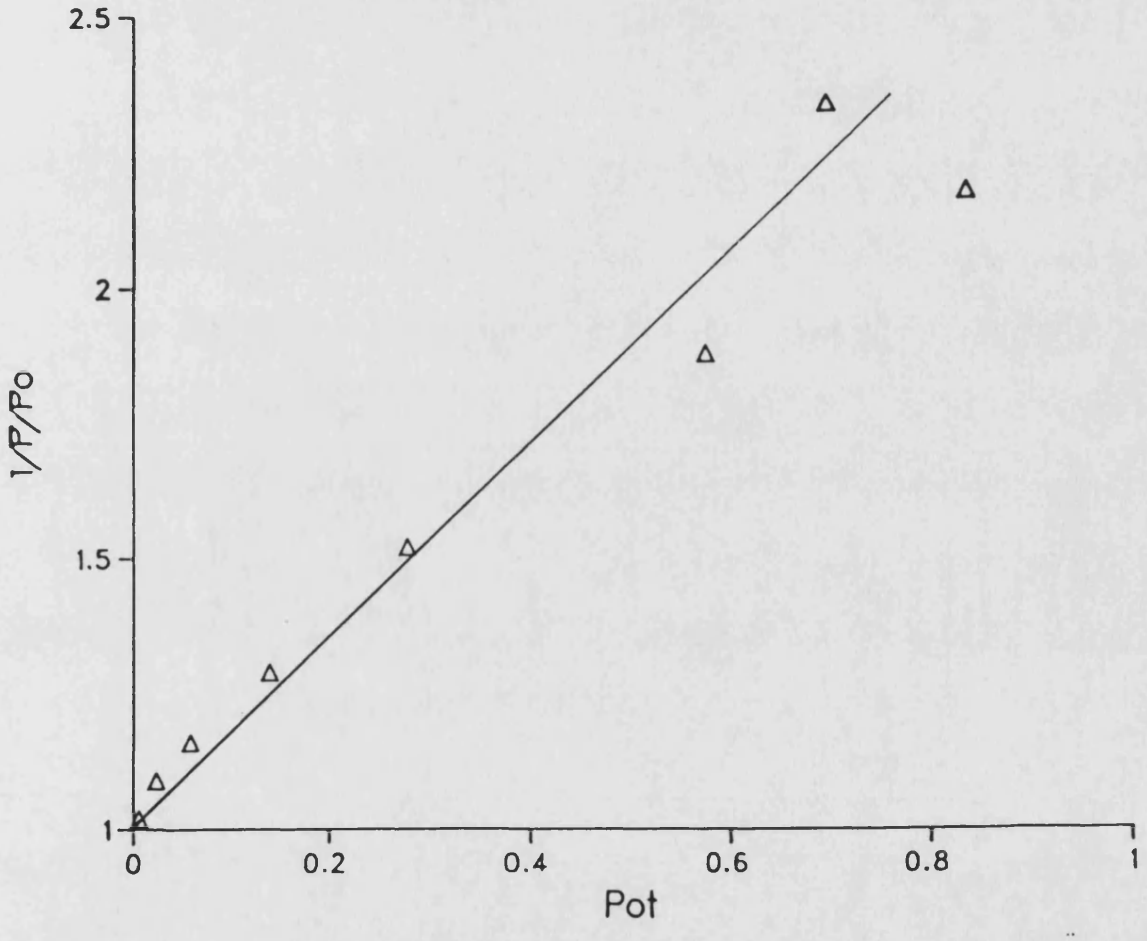


Figure 6.5 The best fit line for the reassociation of denatured probe ct DNA, as determined by linear regression analysis. The data P/P_0 (% single-stranded) and P_0t were expressed in the form $^1/P/P_0 = 1 + kP_0t$.



of probe in the reaction to give pt DNA in µg, from this the amount of probe DNA is subtracted to give the amount of unlabelled pt DNA in the sample which when divided by the total amount of unlabelled DNA added to the reaction gives the percentage pt DNA. The percentage pt DNA estimated for leaf and white tuber tissues are presented in Table 6.1.

Together the percentage pt DNA calculations and the P_0t curves show more pt DNA is detected in the total DNA population of tuber tissue (11.9%) than in leaf tissue (4.2%). Scott *et al.* (1984a) found 7.6% pt DNA in potato leaf tissue and 5.2% pt DNA in tuber tissue (white). The discrepancy between my results and those of Scott *et al.* (1984a) has led to analysis of pt DNA levels in various potato tissues using a different technique, dot blot hybridisation analysis, and different probes.

6.3 Quantification of Plastid DNA Levels in Various Potato Tissues by Dot Blot Hybridisation Analysis

6.3.1 Radiolabelled probe DNA preparation

The plasmid *p*BR322 with the cloned insert of either the sixth Pst I fragment (P6) or the second Bam HI fragment (B2) of wheat ct DNA were used as probes to screen total DNA samples for homologous pt DNA sequences. These probes avoided the complication of nuclear DNA contamination, a problem with the ct DNA probe used in reassociation reactions.

The cloned *p*BR322 DNA was used to transform *E. coli* HB101 cells. The transformants were selected on the basis of Ap^r or Tc^r. Those cells transformed with *p*BR322-P6 conveyed Tc^r, and those cells transformed with *p*BR322-B2 conveyed Ap^r (Figure 6.1). Once selected, the plasmids

Table 6.1 The fraction of pt DNA in total DNA populations from potato leaf and tuber tissues, as determined from linear regression analysis and equation (5):

$$\% \text{ pt DNA} = \{[(P_0t \text{ probe}/P_0t_{\text{total DNA}}) \times 0.5 \text{ µg}] - 0.5 \text{ µg}\}/\text{total DNA (µg)}$$

Tissue	TDNA (µg)	$P_0t_{\frac{1}{2}}$ (moles of nucleotides s l^{-1})	% pt DNA
probe (ct DNA)		0.618	100
leaf	50	0.120	4.2
tuber (white)	50	0.048	11.9

were amplified, in the presence of the appropriate antibotic, and then the cloned *p*BR322 DNA was extracted. To verify that the extracted plasmid DNA actually contained the insert, the cloned fragments were cleaved out of the *p*BR322 DNA with Pst I (*p*BR322-P6) or Bam HI (*p*BR322-B2) restriction enzymes and the resulting fragments were fractionated by agarose gel electrophoresis (Figure 6.6).

The *p*BR322 DNA with the cloned insert was radiolabelled with ^{35}S-CTP by nick-translation (see Section 3.8). Increased incorporation of the radiolabel into DNA was found to be possible by increasing the DNase I/DNA polymerase I and unlablled nucleotide amounts 1.5-fold above the suppliers' recommended amounts. Using the adapted procedure, greater than 30% of the radiolabelled nucleotide was incorporated into the cloned plasmid DNAs; previous attempts gave only 10-20% incorporation. Figure 6.3 shows the typical elution profile of the radiolabelled DNA and residual nucleotides.

6.3.2 Dot hybridisation analysis

Dot hybridisations provide a simple, quantitative means of analysing pt DNA levels in various potato tissues. The principle of hybridisation is the same as for the reassociation reactions, except that the denatured 'driver' DNA (total DNA) in dot hybridisations is immobilised on a membrane instead of being free to move in solution. Pall Biodyne-A nylon membranes were selected as the media on which denatured DNA was immobilised, due to their resistance to cracking or tearing - which I found to be a major problem with nitrocellulose.

DNA concentrations were determined using the 260 nm absorbance criteria and these values were then re-checked using the colorimetric reactions of diphenylamine. A standard curve for the diphenylamine assay was fit using salmon testes sperm DNA as a standard of known

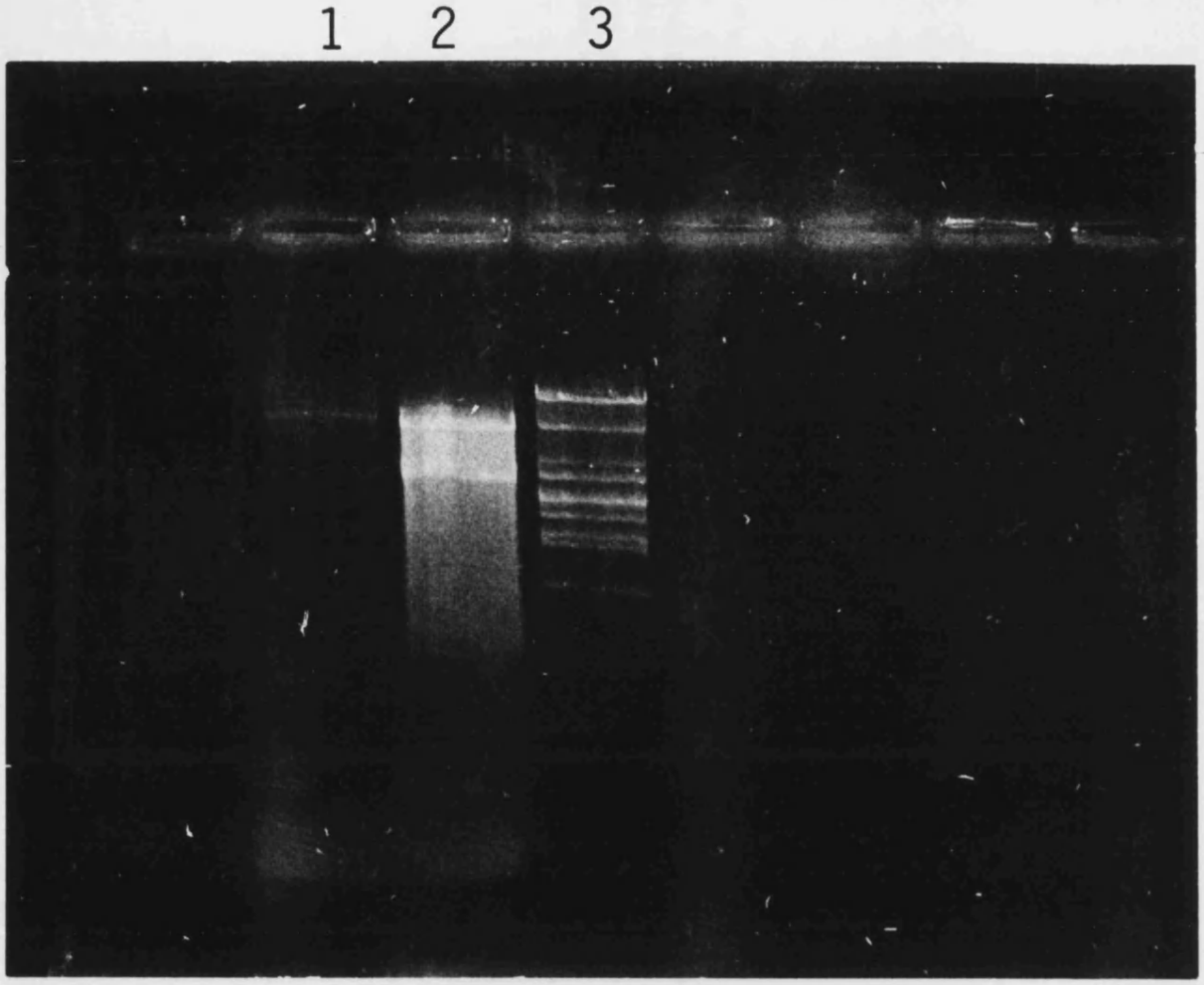


Figure 6.6 Fractionation by agarose gel electrophoreses of the cloned insert DNA and the *p*BR322 DNA after restriction endonuclease digestion. Track 1 shows the 9.6 kbp Bam HI (B_2) wheat ct DNA fragment and the 4.3 kbp *p*BR322 DNA. Track 2 shows the 8.4 kbp Pst I (P_6) wheat ct DNA fragment and the 4 kbp *p*BR322 DNA. λ DNA restricted in a double-digest with Eco RI and Bam HI was used as a marker, the fragments are according to size (kbp) 16.22, additional band due to reannealed cohesive ends, 5.54, 4.74, 3.67, 3.43, 3.33, 2.80, 2.55 and 1.87 from top to bottom.

concentration (Figure 6.7). By interpolation, DNA concentrations of small quantities of potato tissue total DNA or probe DNA samples were determined.

Total DNA samples from potato leaf and tuber - green (4 and 8 days of illumination) and white - tissues were denatured and applied to pre-wet membranes in a dilution series of 0.2, 0.1, 0.05, 0.025 µg per dot. On each filter each dot was in quadruplicate. Denatured, unlabelled *p*BR322-B2 or *p*BR322-P6 were included as standards on all filters according to the radiolabelled probe used. The membranes loaded with the DNA samples were first baked and then allowed to hybridise in the presence of denatured radiolabelled probe. Very stringent washing procedures were necessary to remove any non-specific hybridisation of the probe. The washing procedure recommended for use with the Biodyne-A membranes proved to be the most efficient in removing background, non-specific hybridisation. However, high backgrounds were sometimes found even after prehybridisation with non-homologous salmon sperm DNA and very stringent washing procedures; repeated washing sometimes reduced high backgrounds. Estimates of the fraction pt.DNA in leaf, green tuber and white tuber tissues were made by measuring the hybridisation of probe to each total DNA spot by scintillation counting.

Figure 6.8 shows a typical autoradiograph of dot hybridisation with the radiolabelled *p*BR322-P6 probe. Using the cpm for the individual dots, a curve can be plotted demonstrating the linearity between the quantity of DNA loaded and the amount of hybridisation (Figure 6.9). Membranes were analysed separately to determine the average cpm per μg^{-1} total DNA immobilised on the filter for each potato tissue.

Figure 6.7 Standard curve of salmon testes sperm DNA concentration as measured by spectroscopy (corrected absorbance is $Abs_{595} - Abs_{700}$). This curve was used to determine unknown DNA concentrations.

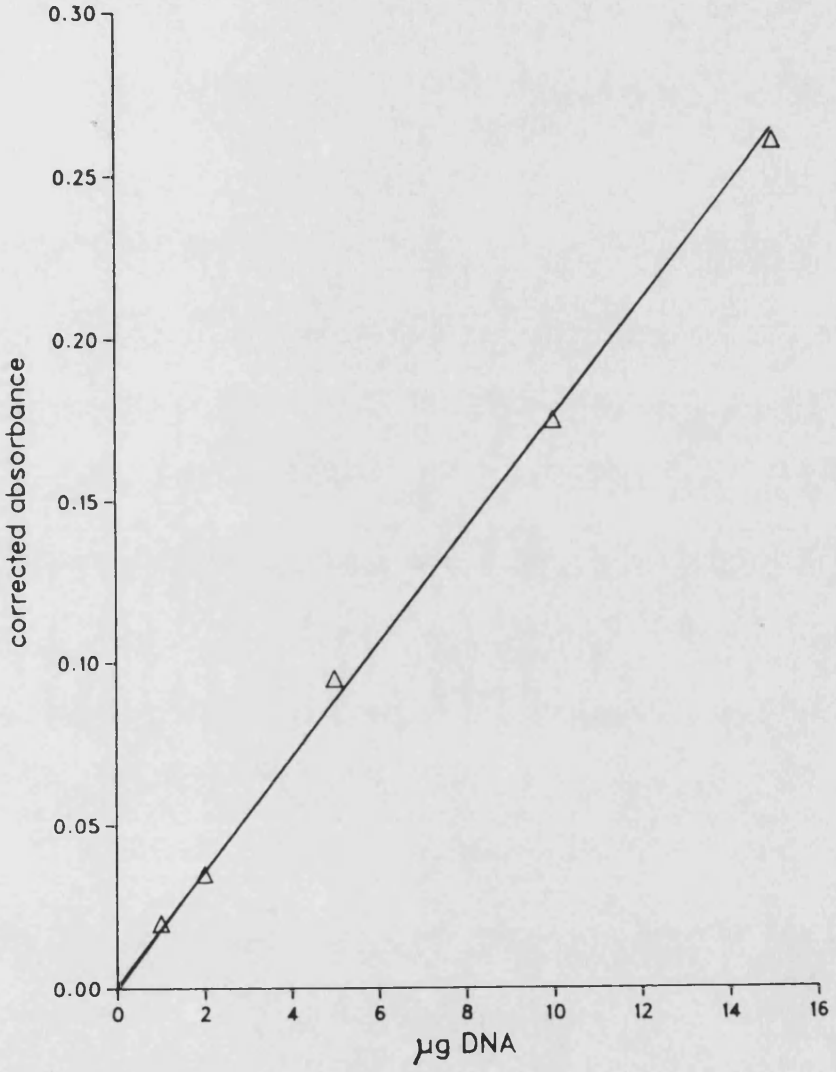


Figure 6.8 Typical dot hybridisations of total DNA (TDNA) from A) leaf tissue, B) green tuber (4 days of illumination) tissue, C) green tuber (8 days of illumination) tissue, and D) white tuber tissue. ^{35}S-CTP-labelled nick-translated *p*BR322-P_6 probe (containing the 16S and 23S rRNA gene sequences) was hybridised to a dilution series of total DNA from various potato tissues. Replicate samples from the same membrane are shown. The radioactive spots were excised from the membrane, and the hybridisation level was determined by scintillation counting.

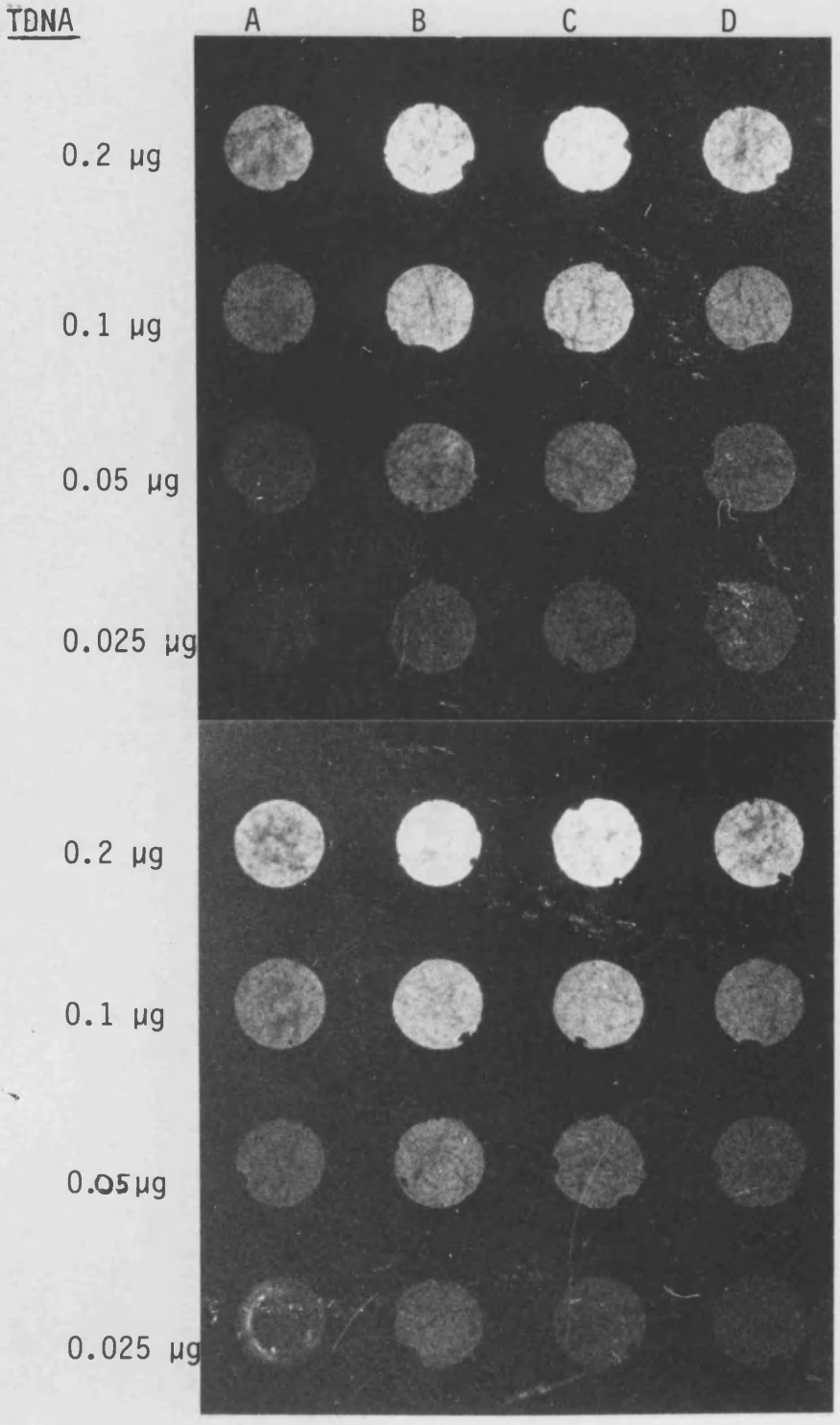
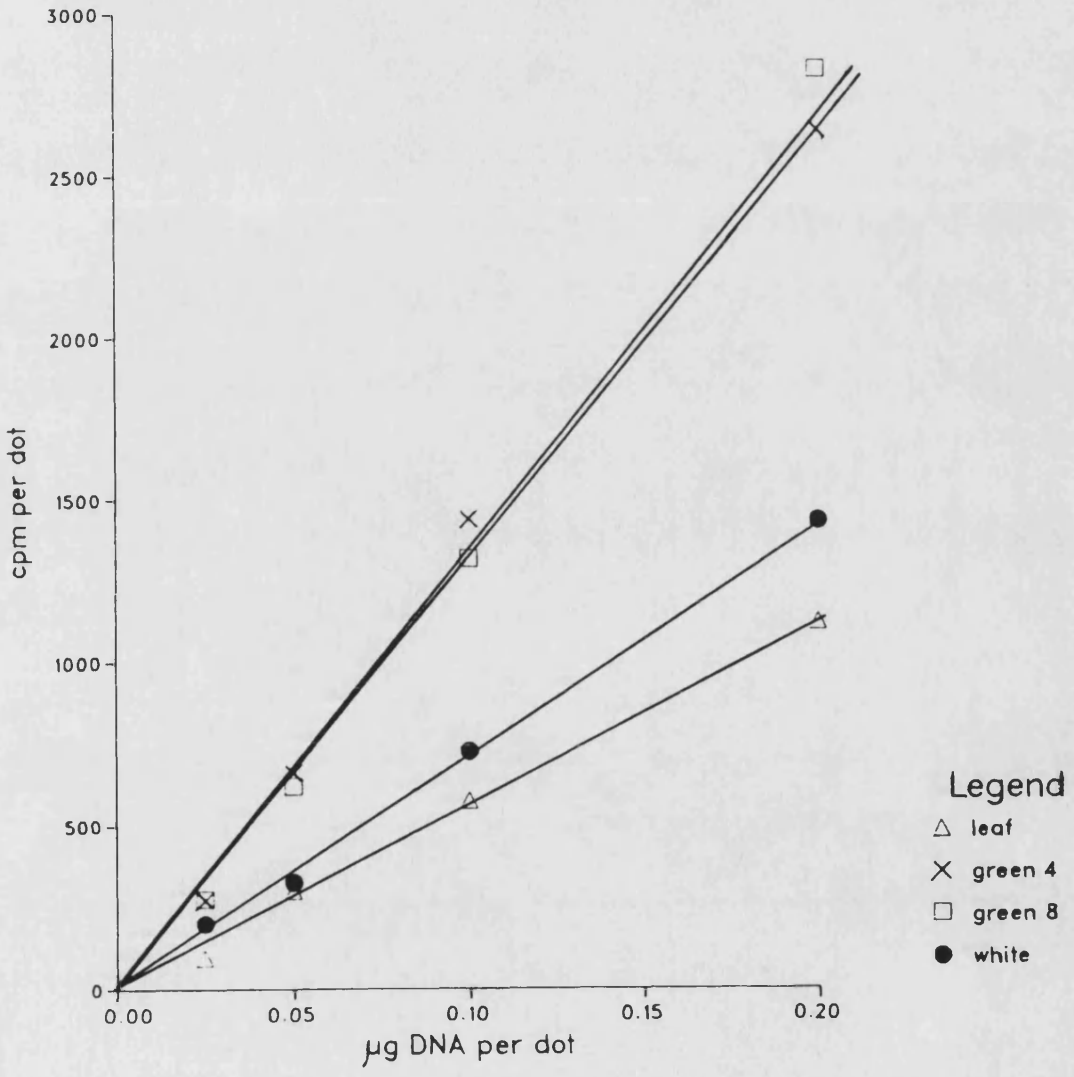

TDNA
A
B
C
D
0.2 µg
0.1 µg
0.05 µg
0.025 µg
0.2 µg
0.1 µg
0.05µg
0.025 µg

Figure 6.9 An example of the linearity found between the dilution series of total DNA applied to Biodyne-A nylon membranes. The cpm for each dilution (*i.e.*, 0.2, 0.1, 0.05 and 0.025 µg DNA) was the mean of 4 replicates from one filter. The trend in percentage pt DNA levels in leaf, green tuber (4 and 8 days of illumination) and white tuber tissues is clearly evident from this example.



The percentage of DNA in the leaf total DNA sample was determined using *p*BR322-P6 as the standard representing 100% hybridisation. Since *p*BR322-P6 represents only 8% of the pt DNA genome, the following equation was used to calculate the percentage pt DNA:

$$[(\text{average cpm leaf } \mu g^{-1} \text{ total DNA})/[(\text{average cpm probe } \mu g^{-1} \text{ total probe DNA}) \times 8\%] = \% \text{ pt DNA in leaf} \quad (1)$$

From the four replicates per filter, the percentage pt DNA in leaf total DNA was determined for each filter. The mean percentage pt DNA of these filters was determined to be 7.1% pt DNA in leaf total DNA. This value was then used to calculate the percentage pt DNA in the other potato tissue total DNA samples:

$$(\text{average cpm}_x \ \mu g^{-1} \text{ total DNA}/\text{average cpm leaf } \mu g^{-1} \text{ total DNA}) \times 7.1\% = \% \text{ pt DNA in x} \quad (2)$$

where x is green or white tuber tissue. Table 6.2 shows the average percentage pt DNA for the same total DNA extraction from replica filters and the average combined percentage pt DNA of the different total DNA extractions.

As found with the reassociation reactions, tuber tissue total DNA shows higher pt DNA levels than leaf tissue, and with greening of the tuber there is an increase in pt DNA level. The variation in percentage pt DNA between filters is most likely due to limitations of the dot hybridisation technique. However, the pattern of hybridisation as detected by the intensity of the dots on the autoradiograph are consistent, with green tuber tissues showing greater hybridisation intensities than white tuber tissue, and leaf tissue showing less intense dots than the tuber tissue.

Table 6.2 Estimates of the percentage pt DNA in total DNA samples from various potato tissues

Tissue	% pt DNA ± S.D. for different total DNA samples[a]					average % pt DNA[b]
	1	2	3	4	5	
leaf[c]	7.1 ± 1.6(3)					7.1%
green (4 days of illumination)	16.3(1)	23.4(1)	11.9(1)	33.8(1)	24.6(1)	22.0% ± 7.5(5)
green (8 days of illumination)	22.8 ± 5.7(3)	19.7(1)				21.3% (2)
white	15.9 ± 6.7(4)	14.4 ± 3.7(4)				15.2% (2)

[a] 1, 2, 3, 4, and 5 represent the different total DNA extractions, and the number of replica filters with the the same total DNA extraction samples are in parentheses.

[b] Number of different total DNA samples analysed in parentheses.

[c] Determined using *p*BR322-P_6 as the known standard (see text).

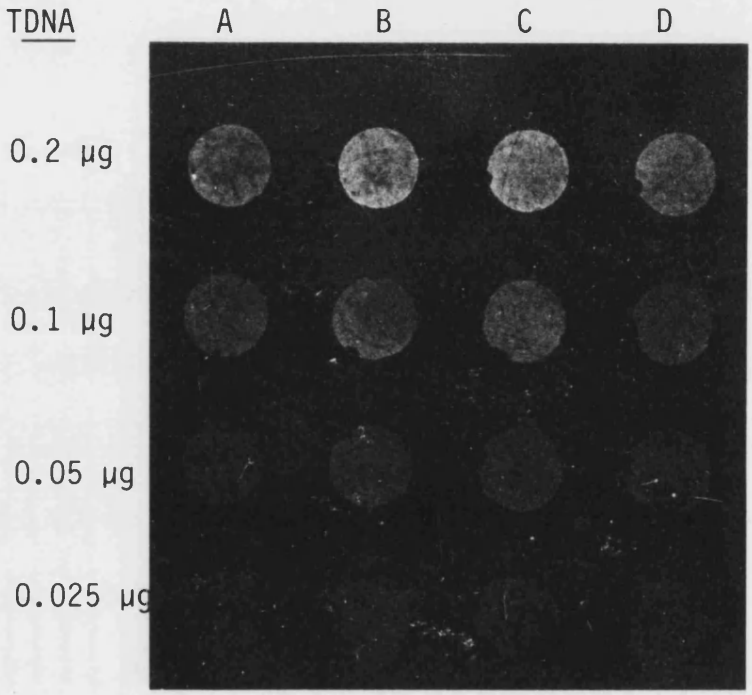
Figure 6.10 shows an autoradiograph of *p*BR322-B2 probe hybridisation to total DNA from various potato tissues. A scan of the autoradiograph showed similar patterns of hybridisation to that found with the *p*BR322-P6 probe. Hybridisation with the *p*BR322-B2 probe proved to be more difficult than hybridisation with the *p*BR322-P6 probe, and this was the only result obtained from several attempts.

6.4 Discussion

Analysis of pt DNA levels in total DNA populations from various potato tissues using the reassociation rate kinetics of radiolabelled ct DNA probe to homologous pt DNA sequences in the total DNA sample showed the pt DNA level found in leaf (4%) was lower than that found in white tuber tissue (12%). These results differed from the 7.6% pt DNA in leaf and 5.2% pt DNA in tuber tissue found by Scott *et al.* (1984a). This led to analysis of pt DNA levels using an alternative method, dot blot hybridisation analysis, with probes containing cloned wheat ct DNA fragments.

The benefits in using dot blot hybridisations were that the hybridisation could be carried out over shorter periods of time and the post-hybridisation washes were of relatively high stringency, both factors favour maximum discrimination for pt DNA sequences in the probe. The dot hybridisation technique also provided a rapid method for analysing replica dilution series of several different samples under exactly the same conditions. An autoradiograph of the post-hybridisation membrane allowed visual assessment of the level of hybridisation of the radiolabelled probe to each dot of immobilised DNA. The linearity found within the dilution series, as determined by scintillation counting, was taken to signify that quantitative immobilisation of the total DNA samples was achieved. This allowed the percentage pt DNA for each sample to be determined quantitatively.

Figure 6.10 Dot hybridisations of total DNA (TDNA) from A) leaf tissue, B) green tuber (4 days of illumination) tissue, C) green tuber (8 days of illumination) tissue, and D) white tuber tissue. ^{35}S-CTP-labelled nick-translated pBR322-B_2 probe (containing the gene sequences rbcL, atpB, atpE and petA) was hybridised to a dilution series of total DNA from various potato tissues. The autoradiograph was scanned using a Joyce-Loebl densitometer.


TDNA
A
B
C
D
0.2 µg
0.1 µg
0.05 µg
0.025 µg

Using the probe *p*BR322-P6 for dot blot hybridisation analysis, 7.1% pt DNA was detected in leaf tissue, a figure very similar to 7.6% pt DNA in leaf obtained by Scott *et al.* (1984a). In tuber tissue high levels of pt DNA were again detected (15%), with an increase in the percentage pt DNA during the greening process (approximately 22%).

In a preliminary study, hybridisation of the *p*BR322-B2 probe in the total DNA samples from leaf, green tuber (4 and 8 days of illumination) and white tuber tissues gave hybridisation patterns which appeared to agree with the patterns obtained using the *p*BR322-P6 probe. However, these experiments need to be repeated for verification.

The use of dot hybridisation analysis seemed to confirm the re-association rate kinetic results that higher pt DNA levels are found in tuber tissue than in leaf tissue. One possible reason for the high pt DNA levels that I found in white tuber tissue by reassociation rate kinetics analysis and dot hybridisation analysis, 12% and 15% respectively, which were not found by Scott *et al.* (1984a), 5.2%, could be the different tissue samples used in experiments. Scott *et al.* used tissue from the whole tuber, whereas I used only the outer parenchyma layers of tissue. Studies were not carried out on the number of plastids per cell or the number of pt DNA copies per plastid in the outer parenchyma tissue of tubers. However, Scott *et al.* (1984a) found relatively few plastids in the tuber cells which they analysed, but estimates of the amyloplast ploidy levels were high (195 pt DNA copies per plastid) compared to chloroplast ploidy levels in leaf (22 pt DNA copies per chloroplast). The high pt DNA ploidy levels found in tuber tissue were suggested to be a fortuitous consequence of the high levels of nuclear ploidy attained in storage cells (Scott *et al.*, 1984a). If this is so, then it would suggest that the cells in the outer layer

of parenchyma tissue have higher nuclear ploidy levels than those in the inner layers or more plastids per cell. As mentioned in the previoous chapter, the average ploidy level for the outer parenchyma layer of white tuber tissue is 23C, which is higher than 14C found by Scott *et al.* (1984a). Depsite this, it must be remembered that high ploidy levels are generally associated with large cell size, and the cells in the inner tissues were found to be larger than those in the outer parenchyma tissues. Further investigations are necessary to determine if the discrepancy is due to the tissue used, the potato variety used, or other environmental/growth factors.

Early studies of pt rRNA genes showed the pt rRNA formed hybrids with both pt DNA and nuclear DNA in *Euglena* (Scott and Smillie, 1967 ; Scott, 1973) and higher plants (Tewari and Wildman, 1968 ; Ingle *et al.*, 1969). The hybrids may have resulted from either cross-homology between the functionally similar rRNA genes in the plastome and the genome, or from the presence of homologous plastid sequences integrated into the nuclear DNA. More recent studies have shown that a large proportion of the spinach pt DNA is homologous to spinach nuclear DNA (Scott and Timmis, 1984b). Scott and Timmis (1984b) estimate the total homology between pt DNA and nuclear DNA to be approximately 5 copies per haploid genome. If similar levels of homology existed between the rRNA genes of the *p*BR322-P6 probe, which contains the 16S and 25S rRNA gene sequences, and the potato nuclear DNA, then the percentage pt DNA content shown in Table 6.2 would be an overestimation. However, this overestimation is probably insignificant in relation to the large number of plastome copies per cell. Scott *et al.* (1984a) found that in their hybridisation study with the rbcL gene that their overestimation was in the order of 15, 70, and 35 plastome copies per cell, for leaf (3000 plastome copies per cell), tuber (7800 plastome

copies per cell), and root (650 copies per cell), respectively. There is also evidence for widespread homologies betwen the plastome and the mitochondrial genome. Stern and Lonsdale (1982) found that corn mt DNA contains a 12kb segment of the corn pt DNA inverted repeat, which includes the rRNA genes. Stern and Palmer (1984) have shown that numerous sequence homologies exist between cloned mung bean and spinach pt DNA restriction fragments and the mt DNAs of corn, mung bean, spinach and pea. Further investigation into the extent of sequence homology between the *p*BR322-P6 probe and the potato mt DNA is required to determine how this might affect the percentage pt DNA determined.

The reason for the high pt DNA levels in white tuber tissue is unclear. Equally unclear is the increase in pt DNA level with greening of the tuber; however, this increase seems to be either directly or indirectly connected to the light-activated transformation of amyloplasts into functional chloroplasts. Though the need for more pt DNA in the greening tissue of potato tuber than in leaf tissue is perplexing, unless, as Scott *et al.* (1984a) suggested, there is some as yet undetermined function of the amyloplasts/chloroplasts of potato tuber.

CHAPTER 7

Qualitative and Quantitative Analysis of Potato Plastid RNA

7.1 Introduction

The advent of 'greening' in potato tuber tissues is the result of a wide variety of metabolic and morphological changes induced by light as amyloplasts transform into functional chloroplasts. Zhu *et al.* (1984) demonstrated by ultracentrifugal sedimentation and immuno-precipitation that RuBPcarboxylase is synthesised *de novo* during the greening of potato tuber. They also showed that light-driven protein synthesis in isolated chloroplasts purified from potato tuber discs exposed to light incorporated ^{35}S-methionine into polypeptides, one of which being the large subunit of RuBPcarboxylase. Furthermore, they showed that these chloroplasts gave a high Hill reaction activity demonstrating that the primary event in photosynthesis, the light-activated transfer of electrons resulting in the splitting of water to give O_2, was functional. This suggests that a light-mediated mechanism directly or indirectly controls specific gene expression in chloroplast development, and thus if the control is at the transcriptional level it would follow that the amount of specific gene transcripts involved in chloroplast functions should increase with 'greening' (*i.e.*, mRNA levels increase). However, the control mechanism could lie at the level of translational control, and thus white and green tuber tissues would have the same level of mRNAs, but with differential translation rates. Two techniques were used to compare the level of specific plastid gene transcripts in green and white tuber tissue, using leaf tissue as a reference: (1) analysis of rRNAs by PAGE, and (2) dot hybridisation analysis of specific chloroplast gene probes to total RNA samples.

Ribosomal RNA is a major component of ribosomes and therefore its level in different tissues is of importance in regulating protein synthesis. Plastids contain 70S ribosomes which under appropriate conditions dissociate into a large 50S subunit and a small 30S subunit. The large subunit contains a 23S rRNA with a mol wt of 1.1×10^6 and the small subunit contains a 16S rRNA with a mol wt of 0.56×10^6. These rRNAs differ in size to the cytoplasmic rRNAs,(25S rRNA mol wt 1.3×10^6 and 18S rRNA mol wt 0.70×10^6). These differences in size allow the four rRNA species to be fractionated using PAGE, thus providing an assay for detecting the levels of each rRNA species in leaf, green tuber and white tuber tissues.

The hybridisation of specific gene probes to total RNA samples through dot hybridisation afforded a method by which the level of specific transcripts could be compared between different tissues. Using probes containing the gene inserts for (1) the 16S and 23S rRNAs (*p*BR322-P6); (2) the large subunit of RuBPcarboxylase (rbcL), β and ε subunits of the CF_1 component of ATP-synthase (atpB, atpE), and cytochrome f (petA), (*p*BR322-B2); (3) atpA and atpB (M13-α and -β); and petA (M13-c), changes in the levels of transcription of these important genes in white and green tuber tissues could be determined.

Hybrid-release translation - a technique whereby pt RNAs were selected by hybridisation to ct DNA bound to a membrane, with the subsequent release and translation of the mRNAs in a cell-free system - was considered as a means to select and translate pt mRNAs from potato tuber tissue total RNA samples. One primary consideration in choosing this method was to avoid the necessity of having to obtain intact amyloplasts or chloroplasts from tuber tissue from which pt RNA could be extracted. The mRNAs were translated in a rabbit reticulocyte lysate cell-free system and the protein products were fractionated by SDS-PAGE.

7.2 Electrophoretic Separation of rRNA Molecules on Polyacrylamide Gels

Polyacrylamide gel electrophoresis (PAGE) provides a method for high-resolution separation of rRNA molecules from a total RNA extract. The six high mol wt rRNA species found in plant cells: 25S and 18S cytoplasmic rRNA (mol wt 1.3×10^6 and 0.7×10^6, respectively), 26S and 18S mitochondrial rRNA (mol wt $1.12\text{-}1.16 \times 10^6$ and $0.69\text{-}0.78 \times 10^6$ respectively), and 23S and 16S plastid rRNA (1.1×10^6 and 0.56×10^6 respectively) were effectively separated on dilute gels of 2.4% acrylamide (with bis-acrylamide at 5% of the acrylamide concentration). The mitochondrial rRNA is present at a low percentage of the total rRNA and is not considered further. Due to the instability of the 23S plastid rRNA (Leaver and Ingle, 1971), total RNA extractions and PAGE procedures were performed at 4° C.

Figure 7.1 shows the profiles of total RNA samples from leaf, green tuber (8 days of illumination) and white tuber tissues run on polyacrylamide gels and scanned at 265 nm. The integrated values for the peak areas, using a LDC/Milton Roy C1-10 integrator, are shown in Table 7.1

Since the heavy rRNAs (1.3×10^6 and 1.1×10^6 mol wt) are approximately twice the size of the light rRNAs (0.70×10^6 and 0.56×10^6 mol wt), a 2:1 molecular ratio of heavy rRNA to light rRNA should be found (Leaver, 1982). Instead, higher levels of 18S rRNA were found than would have been predicted, suggesting some fragmentation of the 23S rRNA despite steps to maintain its integrity. Also, very low levels of 16S rRNA were found. In spite of this, these gels do verify the presence of high mol wt cytoplasmic and plastid rRNAs in potato leaf and tuber tissues.

Figure 7.1 Fractionation of total RNA by polyacrylamide gel electrophoresis. Total RNA samples from leaf, green tuber (8 days of illumination), and white tuber tissues gave 4 major peaks representing the 25S, 23S, 18S and 16S rRNA species. The enriched ct RNA sample also gave 4 peaks, with the ct rRNAs representing the dominant peaks.

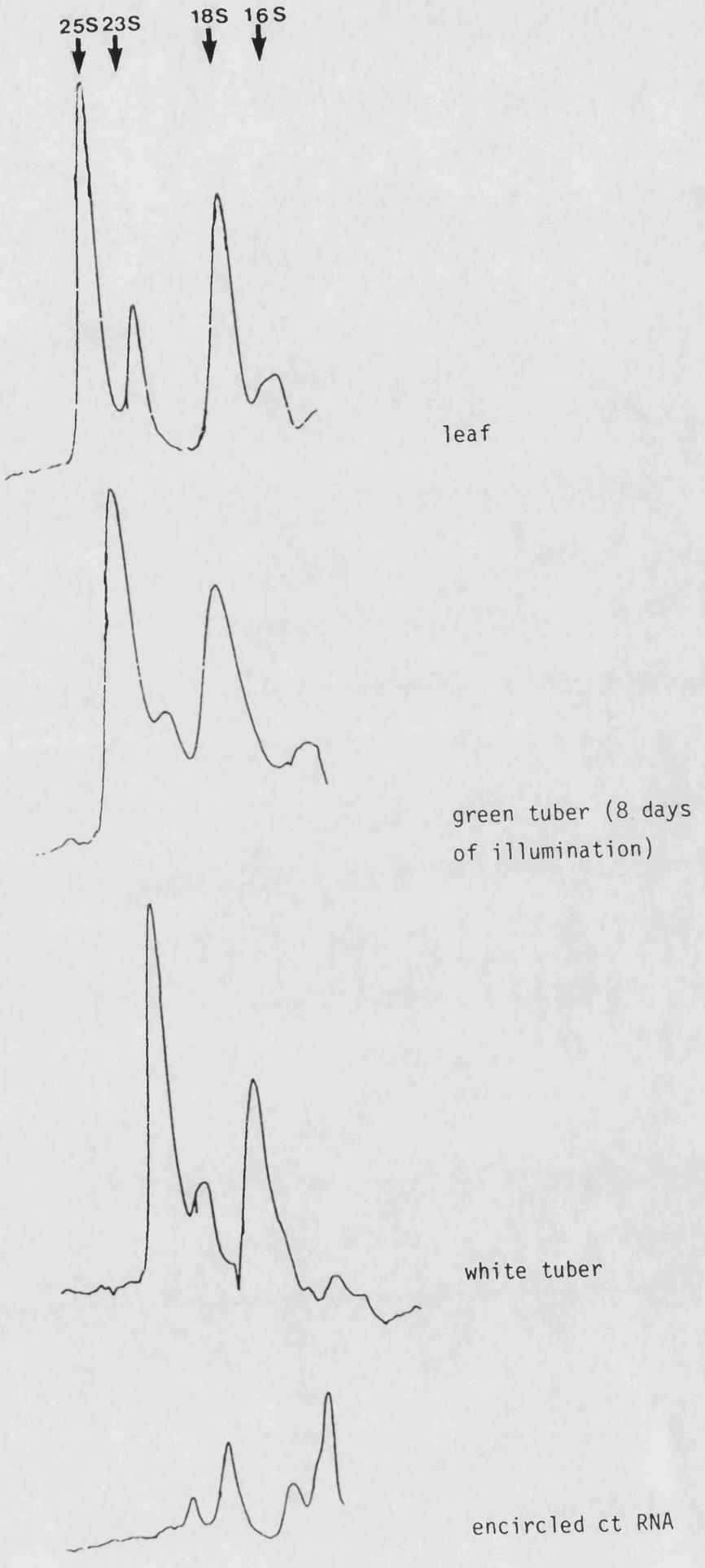

25S
23S
18S
16S
leaf
green tuber (8 days of illumination)
white tuber
encircled ct RNA

Table 7.1 From Figure 7.1 the integrated peak values of the cytoplasmic and chloroplast rRNAs were determined using a LDC/Milton Roy C1-10 integrator. Each peak area is given as a percentage of the total peak area.

	% of Total Peak Area			
Tissue	25S	23S	18S	16S
leaf	43	17	32	8
green (8)	45	11	40	3
white*	48	14	33	2

*Minor peaks account for the remaining 3% of the total peak area

7.3 Dot Blot Hybridisation Analysis of Total RNA Samples using Specific Gene Probes

7.3.1 Probe preparation

The cloned *p*BR322 probes (*p*BR322-B2 and *p*BR322-P6) were selected as in Section 6.3.1. These probes were radiolabelled with ^{35}S-CTP to a high specific activity of 1-10 x 10^8 cpm μg^{-1} DNA by nick-translation.

Wheat ct DNA restriction enzyme fragments containing the gene sequences atpA, atpB and petA were cloned into the β-galactosidase gene fragment of the coliphage M13 DNA. The *E. coli* cells infected by the recombinants were detected by a test for β-galactosidase activity, using the blue/white plaque assay adopted for this vector system. Figure 7.2 shows fractionation of the cloned RF M13 DNA by agarose gel electrophoresis. The cloned M13 DNAs were radiolabelled to a specific activity of 10^7 cpm μg^{-1} DNA with ^{35}S-CTP, higher specific activities were not obtainable.

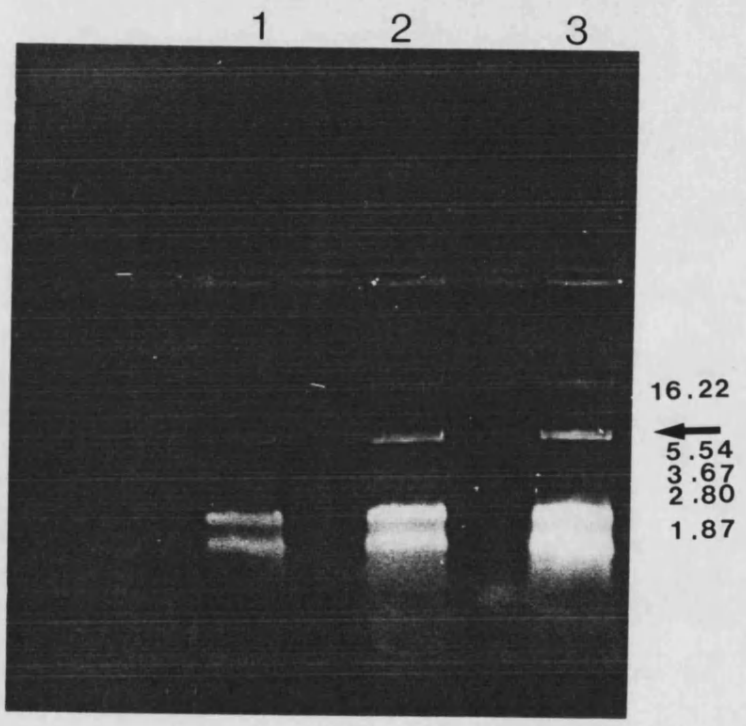
7.3.2 Dot hybridisation analysis

The method for binding RNA to Biodyne-A nylon membranes was according to the procedure recommended by the manufacturers of the membranes. The glyoxal method, described by Thomas (1983) for binding RNA to nitrocellulose, was initially used, but proved unsatisfactory due to extensive spreading of the RNA sample after application. Direct application of total RNA samples to a membrane under light vacuum pressure afforded a reliable and easy procedure with which to bind RNA, without the sample spreading.

The cloned gene probes listed above were used in an attempt to determine the level of transcription of the specific gene sequences

in various potato tissues. However, only the *p*BR322-P6 probe, containing the 16S and part of the 23S rRNA genes, hybridised to total RNA samples to a detectable level. Figure 7.3 shows *p*BR322-P6 hybridisation to total RNA samples from leaf, green tuber (4 and 8 days of illumination) and white tuber tissues. From the autoradiograph there

Figure 7.2 Agarose gel electrophoresis of coliphage M13 DNA plus the atpA (track 1), atpB (track 2) and petA (track 3) gene inserts. The M13 DNA plus insert is approximately 7 kbp and is marked by the arrow. The slow migrating, very faint band is most likely contaminating *E. coli* DNA, and the two fast migrating bands are probably rRNA present in the extraction. The marker is λ DNA digested in a double-digest with EcoRI and Bam H1, the units are in kbp (not all of the marker fragments are shown).



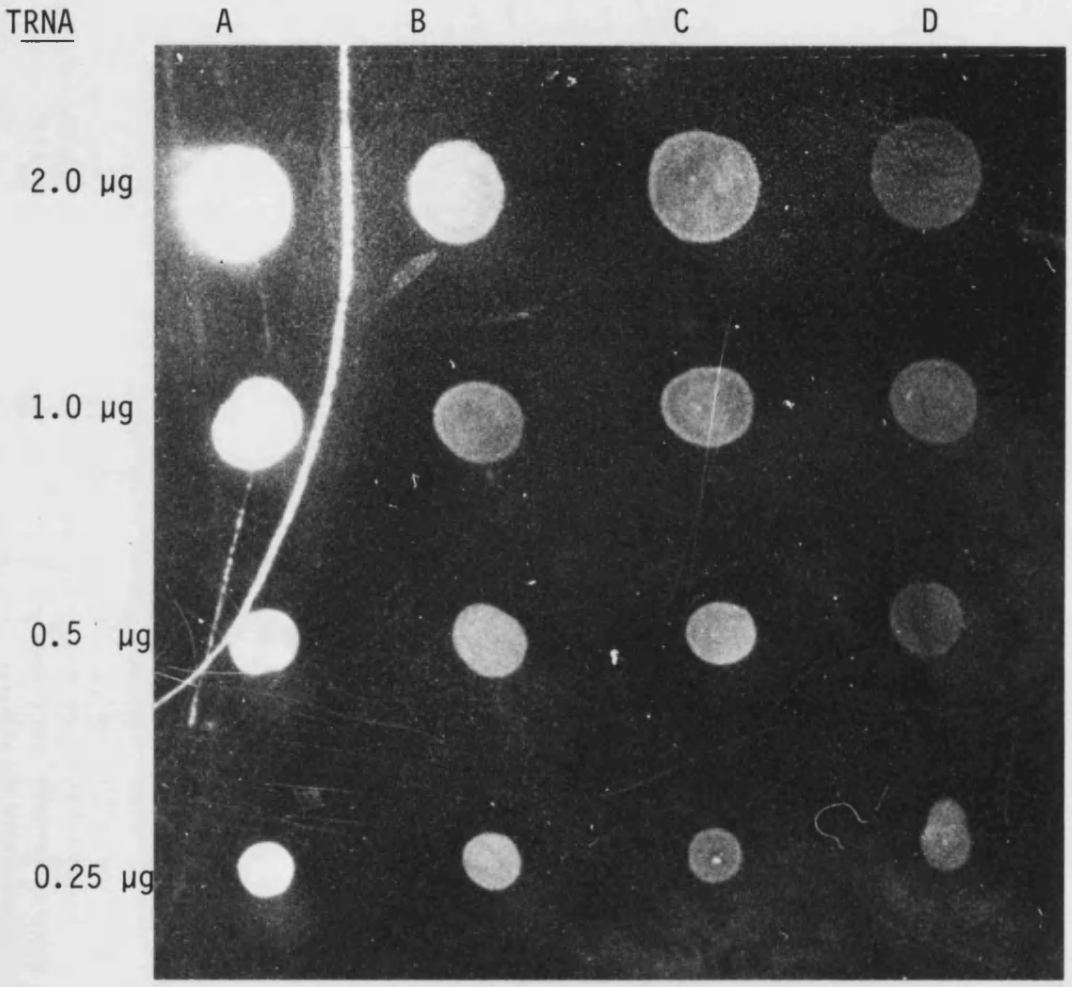


Figure 7.3 Dot hybridisation of ^{35}S-labelled probe (*p*BR322-P_6) containing the 16S and 23S rRNA gene sequences to total RNA samples from A) leaf, B) green tuber (4 days of illumination), C) green tuber (8 days of illumination), D) white tuber (0 days of illumination) tissues. Total RNA (TRNA) was applied to the nylon membrane in decreasing amounts; 2.0 µg, 1.0 µg, 0.5 µg, 0.25 µg.

was only a very low level of hybridisation of the probe to the total RNA samples from white potato tuber tissue and densitometer scans (Joyce Loebl Scanning Densitometer) revealed that the level of hybridisation was only about 1/10 that found with leaf tissue total RNA. The onset of greening of the tuber (4 days of illumination) appeared to cause an increase in rRNA levels to about 1/4 that found in leaf tissue total RNA. Further greening of the tuber (8 days of illumination) did not result in any further increases in rRNA levels, instead a slight decrease in level was found.

Therefore, in agreement with the findings in the previous section (7.2), plastid 23S and 16S rRNAs were found in both leaf and tuber tissues, albeit at relatively low levels in white tuber tissue. Greening of the tuber was accompanied by a rise in the rRNA level, followed by a slight decrease with further illumination.

7.4 Hybrid-Release Translation of Chloroplast mRNA

Hybrid-release translation was the procedure chosen for the identification of pt mRNA protein products from leaf, green, and white tuber tissue total RNA samples. In order to test the applicability of this procedure, bound ct DNA was used to select for ct RNAs from a partially purified ct RNA sample (Section 3.4.3), with the subsequent translation of the ct mRNAs in a rabbit reticulocyte lysate cell-free translation system. The level of protein synthesis and the molecular weights of the translated proteins produced using the hybrid-release ct RNA sample were compared to those obtained from the translation of a leaf total RNA sample, an enriched ct RNA sample (Section 3.4.3) and a control containing no RNA. The radiolabelled translation products from these samples were first analysed by TCA precipitation to determine the level

of protein synthesis, and then by SDS-PAGE to determine the size of the translated protein products.

7.4.1 Level of translation as determined by TCA precipitation

Radiolabelled TCA precipitable proteins were assayed at various time intervals during the mRNA translation reaction. Figure 7.4 shows the incorporation of ^{35}S-methionine into TCA precipitable proteins with time. Analysis of TCA precipitable proteins thus provides a preliminary indication of the level of radiolabel incorporation into TCA precipitable protein products by each of the RNA samples.

7.4.2 Analysis of the radiolabelled protein products by SDS-PAGE

The maximal quantitative translation as determined by TCA precipitation does not necessarily indicate optimal qualitative translation of mRNAs. For this reason, the translation reaction mixture was also assayed by SDS-PAGE and a fluorograph of the gel was used to detect the radiolabelled proteins of mRNA translation.

Figure 7.5 shows a fluorograph of the ^{35}S-labelled proteins produced by the translation system and separated by SDS-PAGE. As expected, leaf total RNA gave a wide range of protein products, which coincided with the highly radioactive TCA precipitate results. The hybrid-release RNA gave one protein band of high mol wt (>45K), a smear of low mol wt protein products (14-18 K) and a smear of very low mol wt protein products (<14 K). The ct-enriched RNA gave two protein bands, one of similar mol wt to the hybrid-release RNA high mol wt band and a band of mol wt between 24-45 K. All tracks showed a radioactive band representing background activity at approximately 24K. This is probably due either to the binding of the ^{35}S-methionine to one of the lysate proteins or due to the translation of endogenous mRNA in the lysate

Figure 7.4 Incorporation of ^{35}S-methionine into TCA precipitable proteins. Total RNA from leaf tissue, RNA resulting from the hybrid-release technique (see text), and an enriched ct RNA (see 3.4.3) were translated in a rabbit reticulocyte lysate system in the presence of ^{35}S-methionine. A sample containing no RNA was used as a control.

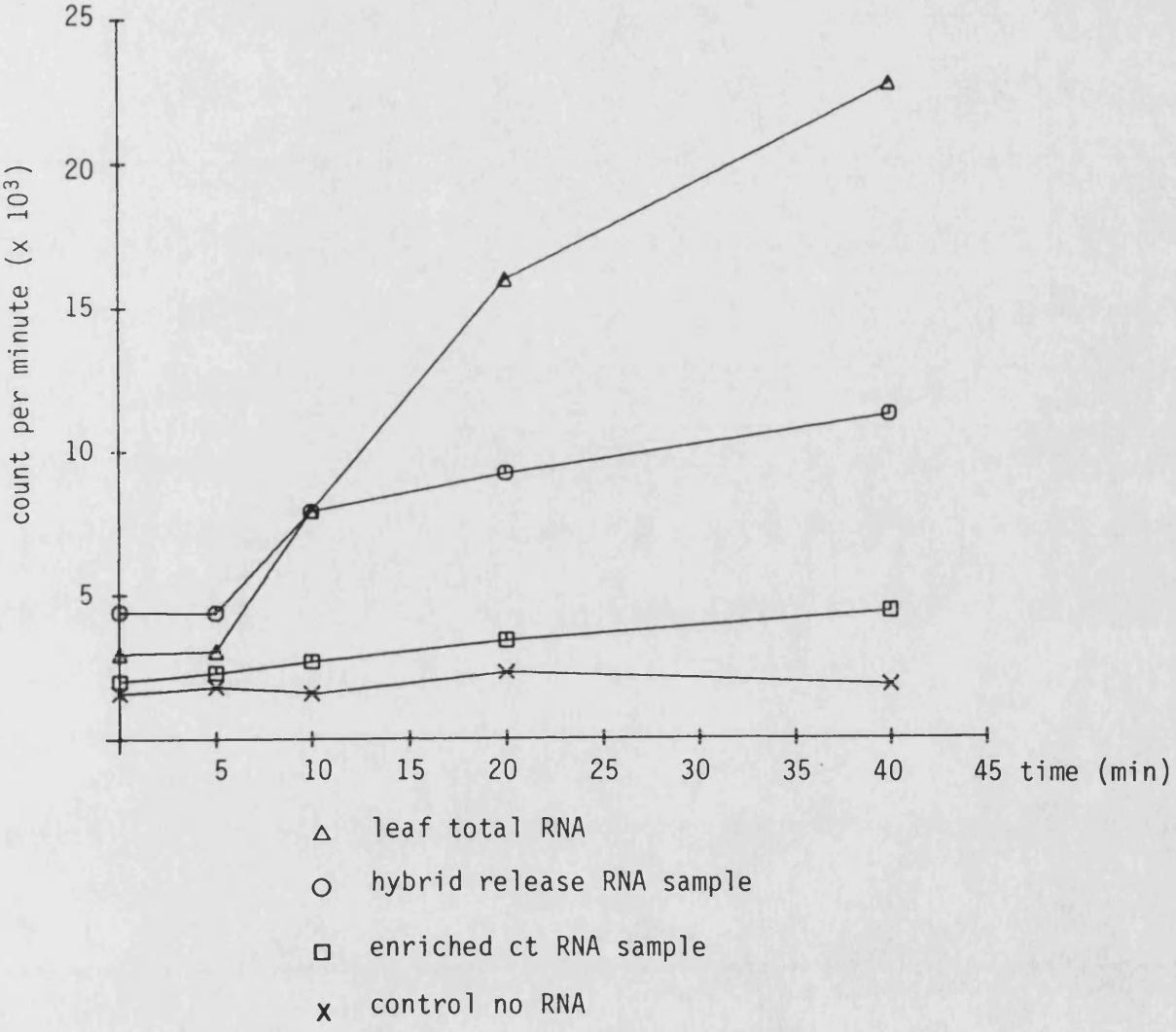
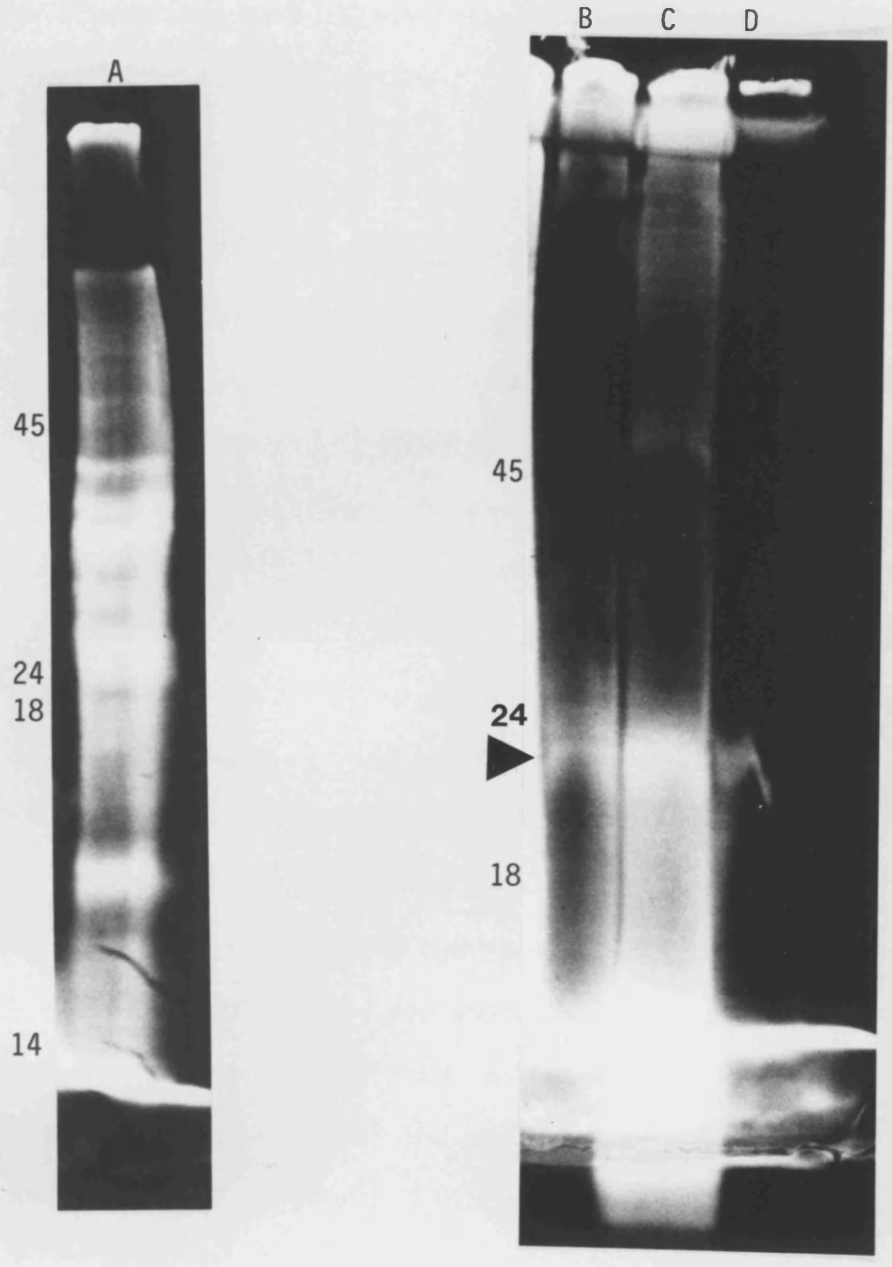


Figure 7.5 Fluorogram of the radiolabelled translation products of A) total leaf RNA, B) enriched ct RNA, C) hybrid release RNA, and D) a control with no RNA. The translated proteins were run on an SDS-polyacrylamide gel with a 7.4% acrylamide stacking gel and a 10% acrylamide separating gel. The molecular weight markers are indicated ($x10^3$). A background band found in the control track and the other tracks is indicated by the solid triangle.


A
45
24
18
14
B
C
D
45
24
18

preparation - this, however, is unlikely due to prior treatment of the lysate with micrococcal nuclease. Due to the low level of translation using the hybrid-release mRNA from partially purified leaf ct RNA, no further attempt was made to use this technique for selecting mRNA from potato tuber total RNA samples.

7.5 Discussion

Plastid rRNA can be separated from cytoplasmic rRNA by means of PAGE. The 23S plastid rRNA is labile in many plant species, but its integrity can be maintained if extraction and fractionation procedures are carried out at temperatures below 5°C (Leaver, 1973; Wollgiehn and Parthier, 1980). If fragmentation does occur, it frequently cleaves the 23S rRNA molecule in three particular regions to produce: $0.7 \times 10^6 + 0.4 \times 10^6$ mol wt components, $0.9 \times 10^6 + 0.2 \times 10^6$ mol wt components or a single $0.5 \times 10^6 - 0.6 \times 10^6$ mol wt product from a median cleavage (Leaver and Ingle, 1971). Leaver (1982) suggested that the fragmentation is due to specific endogenous ribonucleases which attack the exposed loops of the 23S rRNA at the surface of the large ribosomal subunit. Low temperature encourages hydrogen bonding and/or stabilises the secondary helical structure, and therefore preserves the integrity of the 23S rRNA. However, under conditions which disrupt hydrogen bonding (*e.g.*, high temperatures) the secondary structure of the RNA molecule is lost and the molecule dissociates into fragments (Leaver, 1982). In my work, the high levels of 18S cytoplasmic rRNA (mol wt 0.7×10^6) suggest some fragmentation of the 23S rRNA. The presence of very low levels of 16S pt rRNA (mol wt 0.56×10^6) is unexplained, since this rRNA species is known to be stable (Wollgiehn and Parthier, 1980). These gels do, however, demonstrate that both cytoplasmic (25S and 18S) and pt (23S and 16S) rRNA species are present in white and green tuber tissues.

Analysis of probe *p*BR322-P6 (containing the gene sequences for 16S and 23S rRNA genes) hybridisation to total RNA samples from leaf, green tuber and white tuber tissues also showed the presence of plastid high mol wt rRNA components. White tuber tissue total RNA was found to have approximately 1/10 the level of pt rRNA found in leaf tissue total RNA samples, whereas green (4 days of illumination) tuber tissue total RNA had approximately 1/4 the level found in leaf tissue. Further greening showed a slight decrease in pt rRNA level. These results show a sudden rise in pt rRNA level in tuber tissue with the onset of greening, and suggest an increase in translational activity with the onset of greening. Contrary to the findings of Lobov and Bondar (1977), plastid rRNA was found in white tuber tissue, although in low levels. This suggests that amyloplasts may be functional in protein synthesis.

Unfortunately, hybridisation of the cloned M13 probes to total RNA samples did not show detectable levels of hybridisation. This inability to obtain detectable levels of hybridisation with the M13-α, -β, and -c probes could be due to their low specific activities (approximately 10^7 cpm μg^{-1} DNA), compared to the high specific activities of the cloned *p*BR322 probes (approximately 1-10 x 10^8 cpm μg^{-1} DNA). However, the *p*BR322-B2 probe which was radiolabelled to approximately the same specific activity as the *p*BR322-P6 probe did not show detectable levels of hybridisation to total RNA samples. The *p*BR322-B2 probe contains the gene sequence for one of the major protein products found in chloroplasts, the large subunit for RuBPcarboxylase, as well as the gene sequences for petA, atpB and atpE. One possible explanation for the low level of hybridisation with the *p*BR322-B2 probe could be due to the probed sequences representing only a small proportion of the total RNA sample. The total RNA sample contained cytoplasmic as well as plastid RNAs, and

the majority of the transcription products of pt DNA are rRNAs (Wolleighn and Parthier, 1980). Thus, the mRNAs might represent too small a fraction of the total RNA population to be detectable by hybridisation.

A hybrid-release translation system was developed as a possible means for obtaining pt mRNA from potato tissues and determining the protein products resulting from the translation of these pt mRNAs. The mRNAs were translated in a rabbit reticulocyte lysate system, pre-treated with micrococcal nuclease to destroy any endogenous mRNAs. The ability of a eukaryotic system to translate plastid mRNA demonstrates that the constraints which exist *in vivo* and limit chloroplast-directed protein synthesis to 70S ribosomes does not apply to the cell-free translation system. Lerbs *et al.* (1985) found that in the wheat germ cell-free system cytoplasmic mRNAs are more efficiently translated than pt mRNAs and suggested that either (1) the two mRNAs compete for a 'discriminatory initiation factor' independent of the ribosomes, or (2) mRNAs containing 'cap' structures enhance protein synthesis to a greater extent than non-methylated mRNAs, however there is no evidence as to whether pt mRNAs contain 'cap' structures.

Plastid RNA was selected by hybridisation of a partially purified pt RNA sample to membrane-bound ct DNA. The hybridised RNA was released and was then translated in the cell-free translation system. The method was first tested using the hybrid-release RNA, a partially purified pt RNA extract from leaf tissue that had not been further purified by the hybrid-release technique, and a total RNA sample containing cytoplasmic as well as plastid RNAs. From my results, translation of leaf total RNA in the rabbit reticulocyte lysate translation system gave a wide range of protein products. The pt-enriched total RNA sample gave only two protein bands and the hybrid-release pt RNA sample gave only the

high mol wt band found also in the pt-enriched total RNA translation, and low mol wt protein products. The presence of these low mol wt products suggests degradation of the mRNAs before translation. Lerbs *et al.* (1985) found that pt mRNA was more sensitive than cytoplasmic RNA in the extraction procedure to long-term storage and to thawing-refreezing handling, which may explain mRNA degradation. Another possible reason for the low level of translation proucts could be due to the presence of too much non-messenger RNA (*e.g.*, rRNA) which effects translational efficiency (Reisfeld and Edelman, 1982). The hybrid-release RNA samples contained all the RNAs that hybridised to the ct DNA, the majority of which were probably rRNAs.

Future work should concentrate on the fractionation of poly A-RNA from potato tissues by oligo (dT)-cellulose chromatography. This method would avoid the need for selection of pt RNA by hybridisation to ct DNA - a preparation known to contain some nuclear DNA contamination (see Discussion 5.5). It would also avoid the presence of large quantities of rRNA in the preparation. In the wheat germ system poly A-RNA was found to be less stimulatory than poly A+ RNA and gave values of 2-5 fold over background as the upper limit, compared to 20-50 fold stimulation by poly A+ RNA. However, even at these lower levels of stimulation well-defined translation products were produced by the poly A- RNA (Reisfeld and Edelman, 1982). Pelham and Jackson (1976) found that the level of stimulation for translation of RNA in rabbit reticulocyte lysate was 5% that found in the wheat germ system. The reported differences in efficiency of the various translation systems suggests that several cell-free systems should be tried.

CHAPTER 8

General Discussion

One of the most interesting features of the plastids is their ability to interconvert from one form to another. Unlike the primitive plastids of the lower algae, which always retain their thylakoids and are at all times capable of carrying out photosynthesis, many non-photosynthetic plastid forms can be found in higher plants, either in specialised organs, or during specific intervals of the plant's life-cycle. It has been suggested that the proliferation of different types of non-photosynthetic plastids is associated with the evolution of land plants, in particular the angiosperms (Thomson and Whatley, 1980). In tissues where photosynthesis would be ineffective or nonessential, the development of non-photosynthetic plastids may represent an adaption to conserve energy by maintaining plastids in less expensive non-photosynthetic forms (Marinos, 1967), capable of carrying out other metabolic functions. Interestingly, these non-photosynthetic plastids in most cases do retain their plasticity and are able to convert to different plastid forms, dependent on environmental and physiological conditions. The conversion of etioplasts to chloroplasts is extensively documented, much less is known about the development and interconversion of the other plastid forms. In this study, some aspects of the transformation of amyloplasts into chloroplasts were investigated. Several approaches were taken to study the light-induced transformation, each directed towards the further understanding of the function of the plastome/genome interaction and its developmental control.

8.1 The Potato Tuber as a Developmental System

It is well known that potato tubers turn green on exposure to light. The 'greening' indicates the presence of chlorophyll. Phytochrome, through a red light response, is suggested to be the critical factor controlling the transformation of amyloplasts to chloroplasts (Mohr and Oelize-Karow, 1978; Morris *et al.*, 1979). However, Zhu *et al.*, (1984) report that red light does not stimulate amyloplast/chloroplast transformation in tuber tissue, suggesting that phytochrome is not involved. However, the precise mechanism controlling transformation is unclear.

In order to study the transformation of amyloplasts into functional chloroplasts, it is first necessary to define and characterise the tissue chosen for study. Upon illumination, chlorophyll was found to be synthesised only in the peripheral cell layers of tuber tissue. Analysis of cell size in the different regions of whole tubers by light microscopy showed a gradient of decreasing cell size from the large cells in the inner pith to the smaller cells in the peripheral regions. The peripheral cell layers thus provide tissue samples of relatively uniform cell size, capable of chlorophyll synthesis when exposed to light. Using this tissue various molecular aspects of amyloplast to chloroplast transformation could be studied.

8.2 Plastome Reiteration

One intriquing aspect of the plastids is their high degree of plastome reiteration. There are many more copies of the plastome than the genome per cell in leaf tissue (*e.g.*, in potato leaf tissue there are approximately 2500-3000 plastome copies per cell, compared with 4 copies of the genome; Scott *et al.*, 1984a and the results presented here), and even in root tissue the difference is substantial

(*e.g.*, in potato root tissue there are approximately 650 plastome copies, Scott *et al.*, 1984a). This difference is reflected in the number of genes available for transcription and translation into proteins products. In the case of RuBPcarboxylase, where the gene for the large subunit is located in the plastome and the gene for the small subunit is located in the genome, the ratio of cistrons available for transcription can be as high as 1:4000 in pea and 1:800 in spinach (Scott and Possingham, 1982a). This suggests that rapid mRNA turnover or a slow transcription rate in the plastid are possible control factors which necessitate the high plastome copy numbers. However, the ratio of the number of rRNA cistrons available for transcription in the genome and plastome is only 1:2. The reason for the large number of plastome copies per cell is unknown. Speculation suggesting that it conveys stability to the plastome by masking somatic mutations will remain speculations until the number of plastome copies transmitted in the germ line is known. If mutations are masked in the root and leaf where plastome numbers are high, they may be expressed in the germ line if the plastome numbers are very low.

8.3 The Relationship between Nuclear Ploidy and Plastome Levels in Potato Tuber Tissue

Closely co-ordinated activities of the plastome and the genome are necessary in the biogenesis and maintenance of functional plastids. This suggests that a possible regulatory mechanism controls the relative plastome copy numbers to genome ploidy. This possibility was investigated by first determining the nuclear ploidy level in the peripheral tissue of potato tubers and then the percentage pt DNA in the total DNA population. Consistent with the findings of high nulcear ploidy levels in other storage tissues, potato tuber tissue was also found

to have high nuclear ploidy levels, averaging 23C in the peripheral tissue of normal white tubers. Similar levels were also found in tubers exposed to 8 and 12 days of illumination. However, in the tubers exposed to only 4 days of illumination the average nuclear ploidy level fell to 15C. This sharp decrease in ploidy level accompanying the onset of 'greening' was attributed to synchronised cellular division. Light is known to induce plastid division (Cook, 1966), whether it is also responsible for nuclear division in this situation is unknown.

The fixed relationship between chloroplast numbers per cell and the nuclear ploidy of the cell is well-documented in most cell types. In the spongy parenchyma cells of sugar-beet leaves induced to polyploidisation, the chloroplast numbers were closely related to nuclear ploidy levels, with 20-40 chloroplasts in diploid cells and up to 180 in hexaploid cells (Butterfass, 1979). Jellings and Leech (1984) found an almost linear relationship between nuclear ploidy and plastid number in mesophyll cells of *Triticum* and *Aegilops*. Another example showed that in guard cells of a wide range of plant species a doubling of chromosome number is associated with a 70% increase in chloroplast number (Butterfass, 1979). However, the relationship between nuclear ploidy and plastome copy numbers is a bit more tenuous (as discussed in Chapter 6.4). The recent study by Bowman (1986) showed a direct correlation between nuclear ploidy level and plastome levels in mature mesophyll cells of *Triticum* and *Aegilops*. If a similar direct correlation exists between nuclear ploidy and plastome levels in potato tissue, then the percentage pt DNA in total DNA populations from various potato tissues should remain at approximately 7%, as found in leaf tissue, even with increases

in nuclear ploidy. The evidence from my work suggests that a relationship does exist between nuclear ploidy and plastome copy number, but it is not tightly regulated. With the higher ploidy levels, higher plastome levels were observed, but the increase in plastome copy number appeared to be greater than genome increases. With greening of tuber tissue, plastome levels rose with no apparent increase in nuclear ploidy levels. Cannon *et al.* (1985) found that the plastome appeared to be amplified when dark-grown tobacco cells were exposed to light, this most likely occurred through red light activation of phytochrome, which is known to stimulate plastid DNA synthesis (Thompson *et al.*, 1986), although the mechanism of stimulation is elusive. A similar mechanism could be functioning in potato tuber tissue exposed to light, or if phytochrome is not involved then another photoreceptor may be functioning in this regulation. It would be of interest to determine whether plastid number per cell also increased with 'greening' of the tuber, since non-illuminated tuber tissue is known to have low levels of plastids per cell (Scott *et al.*, 1984a). The evidence so far (Zhu *et al.*, 1984) suggests that chloroplasts develop in 'greening' potato tuber tissue by the laying down of photosynthetic membranes in the amyloplasts, thus only a one-for-one conversion may be occurring.

8.4 Gene Expression

The findings of this study only touch the surface of the events of gene expression with plastid transformation. Lobov and Bondar (1977) suggested, based on their findings, that the amyloplasts of potato tuber are incapable of protein synthesis due to the absence of pt rRNAs. Zhu *et. al.*, (1984) found very low levels of protein synthesis in amyloplasts as compared with chloroplasts of non-green and green tuber tissue. My results show low levels

of pt rRNA were found in non-illuminated tuber tissue. This does not verify that protein synthesis is occurring or is even capable of occurring. It does suggest that protein synthesis could be possible if the other components involved in protein synthesis are present and functional. It is not known whether the amyloplast genes are implicated in any function in the potato tuber, therefore their large copy number in amyloplasts of storage tissues may be a wasteful use of cellular energy or there may be some as yet undetermined function requiring large numbers of available cistrons.

With the transformation of the amyloplast into a functional chloroplast active protein synthesis in the amyloplast/chloroplast must occur, since most of the known genes of the plastome are involved in chloroplast functions. This increase in activity was shown with the increased levels of pt rRNA with 'greening' of the tuber. Analysis of the pt mRNAs present at different intervals during plastid transformation as detected by hybridisation to known ct gene probes as well as selective translation of pt mRNA will provide a valuable insight into the regulation mechanism.

8.5 Concluding Remarks

This thesis has undoubtedly posed more questions than it has answered. However, it does illustrate the potential of the potato tuber as a developmental system for further study into photoregulation, plastome gene expression and plastome/genome interaction of plastid transformation.

APPENDIX

In an attempt to obtain a ct DNA preparation free from nuclear DNA contamination the DNase technique described by Herrmann (1982) was used. To avoid ct DNA degradation by DNase, this method requires that the functional integrity of the organelles is maintained.

Unbroken chloroplasts were prepared as described previously (see Section 3.4.1). The pelleted chloroplasts were resuspended in buffer B, in which EDTA was replaced by 0.007 M-$MgSO_4$, made up to 50 ml in a conical tube and kept at 4° C for 1 h. DNaseI was added to the chloroplast suspension to a final concentration of 50-100 μg ml^{-1}. After mixing gently, the suspension was put on ice for 30 min without agitation. It is during this step that the large multi-organelle particles that may entrap nuclear DNA will sediment to the bottom of the conical tube (Herrmann, 1982). The upper two-thirds of the suspension were decanted into approximately 100 ml ice-cold SE buffer [0.15 M NaCl, 0.1 M EDTA (pH 8.0)] and the lower one-third was discarded. The chloroplasts were then pelleted by centrifugation for 5 min at 3000 g at 0° C. The supernatant was discarded. The chloroplast pellet was resuspended in a small volume of SE buffer, diluted 100-fold and washed again. The chloroplasts were lysed and the DNA was extracted as described previously (Section 3.4.2) in the presence of approximately 0.06 M EDTA.

Several attempts using this procedure resulted in very low yields of ct DNA (linear and closed circular). The reason for the low yields could be due to (i) a very low recovery of intact chloroplasts or (ii) DNase penetrating the chloroplast membrane. The former seems unlikely since light microscopy revealed whole intact chloroplasts. However, low ct DNA yields have also been obtained by others using the

DNase technique to extract ct DNA from tomato (Grierson, personal communication) and it has been suggested that the latter reason is a possibility.

REFERENCES

Aitken, A. & Stanier, R.H. (1979) 'Characterisation of peptidoglycan from the cyanelles of *Cyanophora paradoxa*', *J. Gen. Microbiol.* 112, 219-223

Anderson, J.M. & Andersson, B. (1982) 'The architecture of photosynthetic membranes: lateral and transverse organisation', *TIBS* 7, 288-292

Anstis, P.J.P. & Northcote, D.H. (1973) 'Development of chloroplasts from amyloplasts in potato tuber discs', *New Phytol.* 72, 449-463

Arnon, D.I. (1949) 'Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*', *Plant Physiol.* 24, 1-15

Artschwager, E.F. (1924) *J. Agric. Res.* 27, 809-835

Becker, W.M., Leaver, C.J., Weir, E.M. & Reizman, H. (1978) 'Regulation of glyoxysomal enzymes during germination of cucumber', *Plant Physiol.* 62, 542-549

Bedbrook, J.R. & Kolodner, R. (1979) 'The structure of chloroplast DNA', *Ann. Rev. Plant Physiol.* 30, 593-620

Blair, G.E. & Ellis, R.J. (1973) 'Protein synthesis in chloroplasts I. Light-driven synthesis of the large subunit of fraction I protein by isolated pea chloroplasts', *Biochim. Biophys. Acta* 319, 223-234

Blobel, G. (1980) 'Intracellular protein topogenesis', *Proc. Natl. Acad. Sci. USA* 77, 1496-1500

Boardman, N.K. (1966) 'Ribosome composition and chloroplast development in *Phaseolus vulgaris*', *Exp. Cell Res.* 43, 474-482

Bohnert, H.J. & Loffelhardt, W. (1982) 'Cyanelle DNA from *Cyanophora paradoxa* exists in two forms due to intramolecular recombination', *FEBS Lett.* 150, 403-406

Bolivar, F., Rodriguez, R.I., Greene, P.J., Betlach, M.C., Heyneker, H.L., Boyer, H.W., Crosa, J.H. & Falkow, S. (1977) 'Construction and characterisation of new cloning vehicles', *Gene* 2, 95-113

Bonner, W.M. & Laskey, R.A. (1974) 'A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels', *Eur. J. Biochem.* 46, 83-88

Bottomley, W., Spencer, D. & Whitfeld, P.R. (1974) 'Protein synthesis in isolated spinach chloroplasts: Comparison of light-driven and ATP-driven synthesis', *Arch. Biochem. Biophys.* 164, 106-117

Bourque, D.P., Hagiladi, A. & Naylor, A.W. (1973) 'A method for extracting intact chloroplast and cytoplasmic ribosomal RNA from leaves', *Biochem. Biophys. Res. Commun.* 51, 993-999

Bowman, C.M., Koller, B., Delius, H. & Dyer, T.A. (1981) 'A physical map of wheat chloroplast DNA showing the location of the structural genes for the ribosomal RNAs and the large subunit of ribulose 1,5-bisphosphate carboxylase', *Mol. Gen. Genet.* 183, 93-101

Bowman, C.M. & Dyer, T.A. (1982) 'Purification and analysis of DNA from wheat chloroplasts isolated in nonaqueous media', *Anal. Biochem.* 122, 108-118

Bowman, C.M. (1986) 'Copy numbers of chloroplast and nuclear genomes are proportional in mature mesophyll cells of *Triticum* and *Aegilops* species', *Planta* 167, 264-274

Bradbeer, J.W. (1981) 'Development of photosynthetic function during chloroplast biogenesis' in *The Biochemistry of Plants: a Comprehensive Treatis* (Stumpf, P.K. & Conn, E.E., eds.), vol. 8, pp. 423-472, Academic Press, New York

Britten, R.J. & Kohne, D.E. (1968) 'Repeated sequences in DNA', *Science* 161 (3841), 529-540

Burton, K. (1956) 'A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid', *Biochem. J.* 62, 315-322

Butterfass, T.H. (1979) *Patterns of chloroplast reproduction, Cell Biology Monographs* vol. VI, Springer, New York

Cannon, G., Heinhorst, S., Siedlecki, J. & Weissbach, A. (1985) 'Chloroplast DNA synthesis in light and dark grown cultured *Nicotiana tabacum* cells as determined by molecular hybridisation', *Plant Cell Report* 4, 41-45

Chelm, B.K. (1982) 'DNA-DNA renaturation kinetics' in *Methods in Chloroplast Molecular Biology* (Edelman, M. *et al.*, eds.), pp. 301-313, Elsevier Biomedical Press, Amsterdam

Chilton, M., Currier, T.C., Ferrand, S.K., Bendich, A.J., Gordon, M.P. & Nester, E.W. (1974) '*Agrobacterium tumefaciens* DNA and PS8 bacteriophage DNA not detected in Crown Gall tumours', *Proc. Nat. Acad. Sci. USA* 71, 3672-3676

Chu, N.M. & Tewari, K.K. (1982) 'Arrangement of the ribosomal RNA genes in chloroplast DNA of *Leguminosae*', *Mol. Gen. Genet.* 186, 23-32

Chua, N-H. & Gillham, N.W. (1977) 'The sites of synthesis of the principle thylakoid membrane polypeptides in *Chlamydomonas reinhardtii*', *J. Cell Biol.* 74, 441-452

Chua, N-H. & Schmidt, G.W. (1978a) 'Post-translational transport into intact chloroplasts of a precursor to the small subunit of ribulose-1,5-bisphosphate carboxylase', *Proc. Natl. Acad. Sci. USA* 75, 6110-6114

Chua, N-H. & Schmidt, G.W. (1978b) '*In vitro* synthesis, transport, and assembly of ribulose-1,5-bisphosphate carboxylase subunits' in *Photosynthetic Carbon Assimilation* (Siegelman, H.W. & Hind, G., eds.), pp. 325-348, Plenum Press, London and New York

Chua, N-H. & Schmidt, G.W. (1979) 'Transport of proteins into mitochondria and chloroplasts', *J. Cell Biol.* 81, 461-483

Cook, J.R. (1966) 'Studies on chloroplast replication in synchronised *Euglena*' in *Cell Synchrony, Studies in Biosynthetic Regulation* (Cameron, I.L. & Padilla, G.M., eds.), pp. 153-167, Academic Press Inc., New York

Cox, G.C. & Dwarte, D.M. (1981) 'Freeze-etch ultrastructure of a *Prochloron* species - the symbiont of *Didemnum molle*', *New Phytol.* 88, 427-438

Dagert, M. & Ehrlich, S.D. (1979) 'Prolonged incubation in calcium chloride improves the competence of *Esherichia coli* cells', *Gene* 6, 23-28

Davies, D.R. (1976) 'DNA and RNA contents in relation to cell and seed weight in *Pisum sativum*', *Plant Sci. Lett.* 7, 17-25

Davis, R.W., Botstein, D. & Roth, J.R. (1980) *Advanced Bacterial Genetics*, Cold Spring Harbor Laboratory, New York

Dean, C. & Leech, R.M. (1982) 'Genome expression during normal leaf development', *Plant Physiol.* 69, 904-910

Diers, L. (1970) 'Origin of plastids: cytological results and interpretations including some genetical aspects' in *Control of Organelle Development*(Miller, P.L. ed.), 24, 129-146, Symp. Soc. Exp. Biol.

Dobberstein, B., Blobel, G. & Chua, N-H. (1977) '*In vitro* synthesis and processing of a putative precursor for the small subunit of ribulose-1,5-bisphosphate carboxylase of *Chlamydomonas reinhardtii*', *Proc. Natl. Acad. Sci. USA* 74, 1082-1085

Dorne, A.M., Eneas-Filho, J., Heizmann, P. & Mache, R. (1984) 'Comparison of ribosomal proteins of chloroplast from spinach and of *E. coli*', *Mol. Gen. Genet.* 193, 129-134

Ellis, R.J. (1976) 'Protein and nucleic acid synthesis by chloroplasts' in *The Intact Chloroplast* (Barber, J., ed.), pp. 336-359, Elsevier/ North-Holland Biomedical Press, The Netherlands

Ellis, R.J., Smith, S.M. & Barraclough, R. (1980) 'Synthesis, transport and assembly of chloroplast proteins' in *Genome Organisation and Expression in Plants: Proceedings of Nato Advanced Study Institute, Edinburgh, 1979* (Leaver, C.J., ed.), pp.321-335, Plenum Press, New York

Ellis, R.J. (1981) 'Chloroplast proteins: synthesis, transport and assembly', *Ann. Rev. Plant Physiol.* 32, 111-137

Ellis, R.J. (1983) 'Mobile genes of chloroplasts and the promiscuity of DNA', *Nature* 304, 308-309

Falk, H., Liedvogel, B. & Sitte, P. (1974) 'Circular DNA in isolated chromoplasts', *Z. Naturforsch. Teil C* 29, 541-544

Farrelly, F. & Butow, R.A. (1983) 'Rearranged mitochondrial genes in the yeast nuclear genome', *Nature* 301, 296-301

Fluhr, R. & Edelman, M. (1981) 'Conservation of sequence arrangement among higher plant chloroplast DNAs: molecular cross hybridisation among the *Solanaceae* and between *Nicotiana* and *Spinacia*', *Nuc. Acids. Res.* 9, 6841-6853

Fluhr, R., Fromm, H. & Edelman, M. (1983) 'Clone bank of *Nicotiana tabacum* chloroplast DNA: mapping of the alpha, beta, and epsilon subunits of the ATPase coupling factor, the large subunit of ribulosebisphosphate carboxylase, and the 32-kDal membrane protein', *Gene* 25, 271-280

Gellisen, G., Bradfield, J.Y., White, B.N. & Wyatt, G.R. (1983) 'Mitochondrial DNA sequences in the nuclear genome of a locust', *Nature* 301, 631-634

Giddings, T.H., Withers, N.W. & Staehelin, L.A. (1980) 'Supramolecular structure of stacked and unstacked regions of the photosynthetic membranes of *Prochloron sp.*, a prokaryote', *Proc. Natl. Acad. Sci. USA* 77, 352-356

Gillham, N.W. (1978) *Organelle Heredity* Raven, New York

Gillham, N.W., Boynton, J.E. & Chua, N-H. (1978) 'Genetic control of chloroplast proteins', *Curr. Top. Bioenerg.* 8, 211-260

Gray, J.C. & Kecwick, R.G.O. (1974) 'The synthesis of the small subunit of ribulose 1,5-bisphosphate carboxylase in the French bean *Phaseolus vulgaris*', *Eur. J. Biochem.* 44, 491-500

Gray, M.W. & Doolittle, W.F. (1982) 'Has the endosymbiont hypothesis been proven?', *Micro. Rev.* 46, 1-42

Grierson, D. (personal communication).

Gupta, V.S., Gadre, S.R. & Ranjekar, P.K. (1981) 'Novel DNA sequence organisation in rice genome', *Biochim. Biophys. Acta* 656, 147-154

Hagemann, R. & Borner, T. (1978) 'Plastid ribosome-deficient mutants of higher plants as a tool in studying chloroplast biogenesis' in *Chloroplast Development* (Akoyunoglou, G & Argyroundi-Akoyunoglou, J., eds.), pp. 709-720, Elsevier/North-Holland, Amsterdam

Harris, P (1978) 'Triggers, trigger waves, and mitosis: a new model' in *Cell Cycle Regulation* (Jeter *et al.*, eds.), pp. 76-100, Academic Press, London

Heinhorst, S. & Shively, J.M. (1983) 'Encoding of both subunits of ribulose-1,5-bisphosphate carboxylase by organelle genome of *Cyanophora paradoxa*', *Nature* 304, 373-374

Heldt, H.W. (1976) 'Metabolite carriers of chloroplasts' in *Transport in Plants* (Stocking, G.R. & Heber, U., eds.), vol.III, pp. 137-143, Springer, Heidelberg

Herrmann, R.H., Seyer, P., Schedel, R., Gordon, K., Bisanz, C., Winter, P., Hildebrandt, J.W., Wlascher, M., Alt, J. & Sears, B.B. (1980) 'The plastid chromosomes of several dicotyledons' in *Biological Chemistry of Organelle Information* (Bücher, T., *et al.* eds.), pp. 97-112, Springer, New York

Herrman, R.G. (1982) 'The preparation of circular DNA from plastids' in *Methods in Chloroplast Molecular Biology* (Edelman, M. *et al.*, pp. 259-280, Elsevier Biomedical Press, Amsterdam

Highfield, P.E. & Ellis, R.J. (1978) 'Synthesis and transport of the small subunit of chloroplast ribulose bisphosphate carboxylase', *Nature* 271, 420-424

Hildebrand, M., Jurgenson, J.E., Ramage, R.T. & Bourque, D.P. (1985) 'Derivation of a physical map of chloroplast DNA from *Nicotiana tabacum* by two-dimensional gel and computer-aided restriction analysis' *Plasmid* 14, 64-79

Hoober, J.K. (1976) 'Protein synthesis in chloroplasts' in *Protein Synthesis* (McConkey, E.H., ed.), vol. 2, pp.169-248, Dekker, New York

Hoober, J.K. (1984) *Chloroplasts* Plenum Press, London

Howe, C.J., Auffret, A.D., Doherty, A., Bowman, C.M., Dyer, T.A. & Gray, J.C. (1982a) 'Location and nucleotide sequence of the gene for the proton-translocating subunit of wheat chloroplast ATP synthase', *Proc. Natl. Acad. Sci. USA* 79, 6903-6907

Howe, C.J., Bowman, C.M., Dyer, T.A. & Gray, J.C. (1982b) 'Localisation of wheat chloroplast genes for the beta and epsilon subunits of ATP synthase' *Mol. Gen. Genet.* 186, 525-530

Ingle, J., Possingham, J.V., Wells, R., Leaver, C.J. & Loening, U.E. (1969) 'The properties of chloroplast ribosomal RNA' in *Control of Organelle Development* (Miller, P.L., ed.), pp. 303-325, Cambridge University Press, Cambridge

Jacobs, H.T., Posakony, J.W., Grula, J.W., Roberts, J.W., Xin, J-H., Britten, R.J. & Davidson, E.H. (1983) 'Mitochondiral DNA sequences in the nuclear genome of *Strongylocentrotus purpuratus* *J. Mol. Biol.* 165, 609-632

Jadhav, S.J. & Salunkhe, D.K. (1975) 'Formation and control of chlorophyll and glycoalkaloids in tubers of *Solanum tuberosum L.* and evaluation of glycoalkaloid toxicity' *Adv. Food Res.* 21, 307-354

Jellings, A.J. & Leech, R.M. (1984) 'Anatomical variation in first leaves of nine *Triticum* genotypes and its relationship to photosynthetic capacity', *New Phytol.* 96, 371-382

Jenner, C.F. (1974) 'Factors in the grain regulating the accumulation of starch' in *Mechanisms of Regulation of Plant Growth* (Bieleski, R.L. *et al.*, eds.), pp. 901-908, *Bull Roy. Soc. NZ* 12 Wellington

Jenner, C.F. (1982) 'Storage of starch' in *Encyclopaedia of Plant Physiology, N.S.* vol.13A: Plant carbohydrates I. Intracellular carbohydrates (Loewus, F.A. & Tanner, W., eds.), pp. 700-737, Springer, New York

Jones, B.L., Nagabhushan, N., Tucker, E.B. & Zalik, S. (1973) 'Dissociation reassociation and phenylalanine incorporation by chloroplast and cytoplasmic wheat-leaf ribosomes', *Can. J. Biochem.* 51, 686-693

Kirk, J.T.O. & Tilney-Bassett, R.A.E. (1978) *The Plastids. Their Chemistry, Structure, Growth and Inheritance* Elsevier, Amsterdam

Ko, K. Jaynes, J.M. & Straus, N.A. (1985) 'Homology between the cyanelle DNA of *Cyanophora paradoxa* and the chloroplast DNA of *Vicia faba*', *Plant Sci.* 42, 115-123

Koller, B. & Delius, H. (1980) '*Vicia faba* chloroplast DNA has only one set of ribosomal RNA genes as shown by partial denaturation mapping and R-loop analysis', *Mol. Gen. Genet.* 178, 261-269

Kolodner, R. & Tewari, K.K. (1975) 'The molecular size and conformation of the chloroplast DNA from higher plants', *Biochim. Biophys. Acta* 402, 372-390

Kubitschek, H.E. (1974) 'Constancy of the ratio of DNA to cell volume in steady-state cultures of *Escherichia coli* B/r', *Biophys. J.* 14, 119-123

Lamppa, G.K. & Bendich, A.J. (1979) 'Changes in chloroplast DNA levels during development of pea (*Pisum sativum*)', *Plant Physiol.* 64, 126-130

Leaver, C.J. & Ingle, J. (1971) 'The molecular integrity of chloroplast ribosomal ribonucleic acid', *Biochem. J.* 123, 235-243

Leaver, C.J. (1973) 'Molecular integrity of chloroplast ribosomal ribonucleic acid', *Biochem. J.* 135, 237-240

Leaver, C.J. (1982) 'Isolation of intact 16S and 23S ribosomal RNAs from higher plants' in *Methods in Chloroplast Molecular Biology* (Edelman M. *et al.*, eds.), pp. 369-375, Elsevier Biomedical Press, Amsterdam

Ledoigt, G. & Freyssinet, G. (1982) 'Plastid ribosome', *Biol. Cell* 46, 215-238

Lemaux, P. & Grossman, A. (1984) 'Isolation and characterisation of a gene for a major light-harvesting polypeptide from *Cyanophora paradoxa*', *Proc. Natl. Acad. Sci. USA* 81, 4100-4104

Lerbs, S., Wollgiehn, R., Lerbs, W. & Parthier, B. (1985) 'Cell-free translation of chloroplast RNAs in the wheat germ system', *Plant Sci.* 38, 199-205

Lewin, R.A. & Withers, N.W. (1975) 'Extraordinary pigment composition of a prokaryotic alga, *Nature* 256, 735-737

Lobov, V.P. & Bondar, P.I. (1977) 'The RNA of potato tuber amyloplasts', *Fiziol. Rast.* 24, 318-322

Loenen, W.A.M. & Brammar, W.J. (1980) 'A bacteriophage lambda vector for cloning large DNA fragments made with several restriction enzymes', *Gene* 20, 249-259

Loening, U.E. (1967) 'The fractionation of high-molecular-weight ribonucleic acid by polyacrylamide-gel electrophoresis', *Biochem J.* 102, 215-257

Lovell, P.H. & Booth, A. (1967) 'Effects of gibberellic acid on growth, tuber formation and carbohydrate distribution in *Solanum tuberosum*', *New Phytol.* 66, 525-537

Lyttleton, J.W. (1962) 'Isolation of ribosomes from spinach chloroplasts', *Exp. Cell Res.* 26, 312-317

MacKinney, G. (1941) 'Absorption of light by chlorophyll solutions', *J. Biol. Chem.* 140, 315-322

McLeish, J. & Sunderland, N. (1961) 'Measurements of deoxyribose nucleic acid (DNA) in higher plants by Feulgen photometry and chemical methods', *Exp. Cell Res.* 24, 527-540

Madison, J.T., Thompson, J.F. & Muenster, A.E. (1976) 'Deoxyribonucleic acid, ribonucleic acid, protein and uncombined amino acid content of legume seeds during embryogeny', *Ann. Bot.* 40, 745-756

Mandel, M. & Higa, A. (1970) 'Calcium-dependent bacteriophage DNA infection', *J. Mol. Biol.* 53, 159-162

Maniatis, T., Fritsch, E.F. & Sambrook, J. (1982) *Molecular Cloning: a Laboratory Manual* Cold Spring Harbor Laboratories, New York

Mares, D.J. & Marschner, H. (1980) 'Assimilate conversion in potato tubers in relation to starch deposition and cell growth', *Ber. Dtsch. Bot. Ges.* 93, 299-313

Marinos, N.G. (1967) 'Multifunctional plastids in the meristematic region of potato tuber buds', *J. Ultrastruct. Res.* 17, 91-113

Messing, J. (1983) 'New M13 vectors for cloning' in *Methods in Enzymology* (Wu, R., Grossman, L. & Moldave, K., eds.), vol. 101, pp. 20-78, Academic Press, New York

Millerd, A. & Spencer, D. (1974) 'Changes in RNA-synthesising activity and template activity in nuclei from cotyledons of developing pea seeds', *Aus. J. Plant Physiol.* 1, 331-341

Mohr, H. & Oelze-Karow, A. (1978) 'Phytochrome and chloroplast development' in *Chloroplast Development* (Akoyunoglou, G & Argyrondi-Akoyunoglou, J.H., eds.), pp. 769-779, Elsevier/ North-Holland, Amsterdam

Moorby, J. (1978) 'The physiology of growth and tuber yield' in *The Potato Crop* (Harris, P.M., ed.), pp. 153-194, Chapman and Hall, London

Morris, S.C., Graham, D. & Lee, T.H. (1979) 'Phytochrome control of chlorophyll synthesis in potato tubers', *Plant Sci. Lett.* 17, 13-19

Muhlethaler, K. & Frey-Wyssling, A. (1959) 'Entwicklung und struktur der proplastiden', *J. Biophys. Biochem. Cytol.* 6, 507-512

Nagl, W. (1973) 'The mitotic and endomitotic nuclear cycle in *Allium carinatum*', *Chromosoma* 44, 203-212

Newbury, H.J., Sedgley, M. & Possingham, J.V. (1978) 'Nucleic acid metabolism during early development of pollinated and auxin induced partheocarpic watermelon fruits', *J. Exp. Bot.* 108, 207-215

Palmer, J.D. & Thompson, W.F. (1981) 'Rearrangements in the chloroplast genomes of mung bean and pea', *Proc. Natl. Acad. Sci. USA* 78, 5533-5537

Palmer, J.D. & Thompson, W.F. (1982/83) 'A general model for chloroplast genome evolution', *Annual Report, Carnegie Institution,* Washington pp.26-30

Park, W.D. (1984) 'Potato tuber proteins as molecular probes for tuberisation', *HortScience* 19, 37-40

Pelham, H.R.B. & Jackson, R.J. (1976) 'An efficient mRNA-dependent translation system from reticulocyte lysate', *Eur. J. Biochem.* 67, 247-256

Phillips, A.L. (1985a) 'Restriction map and clone bank of tomato plastid DNA', *Curr. Genet.* 10, 147-152

Phillips, A.L. (1985b) 'Localisation of genes for chloroplast components in tomato plastid DNA', *Curr. Genet.* 10, 153-161

Possingham, J.V. (1980) 'Plastid replication and development in the life cycle of higher plants', *Ann. Rev. Plant Physiol.* 31, 113-129

Raff, R.A. & Mahler, H.R. (1972) 'The nonsymbiont origin of mitochondria', *Science* 177, 575-582

Reisfield, A. & Edelman, M. (1982) 'Translation of chloroplast poly A⁻ mRNA in the wheat germ system' in *Methods in Chloroplast Molecular Biology* (Edelman M. *et al.*, eds.), pp. 605-614, Elsevier Biomedical Press, Amsterdam

Sager, R. (1972) *Cytoplasmic genes and Organelles* Academic Press, New York

Sager, R. (1977) 'Genetic analysis of chloroplast DNA in *Chlamydomonas*', *Adv. Genetics* 19, 287-340

Sager, R., Sano, H. & Grabowy, C.T. (1984) 'Control of maternal inheritance by DNA methylation in *Chlamydomonas*', *Curr. Top. Microbiol. Immunol.* 108, 157-173

Scharpe, A. & van Parijs, R. (1973) 'The formation of polyploid cells in ripening cotyledons of *Pisum sativum L.* in relation to ribosome and protein synthesis', *J. Exp. Bot.* 24, 216-222

Schiller, B., Herrmann, R.G. & Melchers, G. (1982) 'Restriction endonuclease analysis of plastid DNA from tomato, potato and some of their somatic hybrids', *Mol. Gen. Genet.* 186, 453-459

Schmidt, G.W., Bartlett, S., Grossman, A.R., Cashmore, A.R. & Chua, N-H. (1980) '*In vitro* synthesis, transport and assembly of the constituent polypeptides of the light-harvesting chlorophyll a/b protein complex', in *Genome Organisation and Expression in Plants* (Leaver, C.J., ed.), pp. 337-351, Plenum Press, London

Schwarz, Z. & Kössel, H. (1980) 'The primary structure of 16S rDNA from *Zea mays* chloroplasts is homologous to *E. coli* 16S rRNA', *Nature* 283, 739-742

Scott, N.S. & Smillie, R.M. (1967) 'Evidence for the direction of chloroplast ribosomal RNA synthesis by chloroplast DNA', *Biochem. Biophys. Res. Commun.* 28, 598-603

Scott, N.S. (1973) 'Ribosomal RNA cistrons in *Euglena gracilis*', *J. Mol. Biol.* 81, 327-336

Scott, N.S. & Possingham, J.V. (1980) 'Chloroplast DNA in expanding spinach leaves', *J. Exp. Bot.* 123, 1081-1092

Scott, N.S. & Possingham, J.V. (1982a) 'Leaf development' in *The Molecular Biology of Plant Development* (Smith, H. & Grierson, D., eds.), pp. 223-255, Blackwell, Oxford

Scott, N.S., Cain, P. & Possingham, J.V. (1982b) 'Plastid DNA levels in albino and green leaves of the 'albostrians' mutant of *Hordeum vulgare*', *Z. Pflanzenphysiol. Bd.* 108, 187-191

Scott, N.S. & Possingham, J.V. (1983) 'Changes in chloroplast DNA levels during growth of spinach leaves', *J. Exp. Bot.* 34, 1756-1767

Scott, N.S., Tymms, M.J. & Possingham, J.V. (1984a) 'Plastid DNA levels in the different tissues of potato', *Planta* 161, 12-19

Scott, N.S. & Timmis, J.N. (1984b) 'Homologies between nuclear and plastid DNA in spinach', *Theor. Appl. Genet.* 67, 279-288

Seyer, P., Kowallik & Herrmann, R.G. (1981) 'A physical map of *Nicotiana tabacum* plastid DNA including the location of structural genes for ribosomal RNAs and the large subunit of ribulose bis-phosphate carboxylase/oxygenase', *Curr. Genet.* 3, 189-204

Shen, C.F., Chen, K., Wu, M. & Kung, S.D. (1982) '*Nicotiana* chloroplast genome. 4. *N. acuminata* has larger inverted repeats and genome size', *Mol. Gen. Genet.* 187, 12-18

Shinozaki, K., Deno, H., Sugita, M., Kuramitsu, S. & Sugiura, M. (1986) 'Intron in the gene for the ribosomal protein S16 of tobacco chloroplast and its conserved boundary sequences', *Mol. Gen. Genet.* 202, 1-5

Siegel, A. (1974) in *Modification of the Information Content of Plant Cells* Proc. Sec. John Innes Symp. (Makken, R., *et al.*, eds.), pp. 15-26, Elsevier/North Holland, Amsterdam

Slater, J.W. (1963) 'Mechanisms of tuber initiation' in *The Growth of the Potato* pp. 114-131, Butterworth, London

Smith, S. & Ellis, R.J. (1979) 'Processing of the small subunit precursor of ribulose bisphosphate carboxylase and its assembly into whole enzymes are stromal events', *Nature* 278, 662-664

Stern, D.B. & Lonsdale, D.M. (1982) 'Mitochondrial and chloroplast genomes of maize have a 12-kilobase DNA sequence in common', *Nature* 299, 698-702

Stern, D.B. & Palmer, J.D. (1982/83) 'Widespread presence of chloroplast DNA sequences in plant mitochondrial genomes', *Annual Report, Carnegie Institution,* Washington, 17-19

Stern, D.B. & Palmer, J.D. (1984) 'Extensive and widespread homologies between mitochondrial DNA and chloroplast DNA in plants', *Proc. Natl. Acad. Sci. USA* 81, 1946-1950

Sugita, M. & Sugiura, M. (1983) 'A putative gene of tobacco chloroplast coding for ribosomal protein similar to *E. coli* ribosomal protein S19', *Nucl. Acids Res.* 11, 1913-1918

Sugita, M., Kato, A., Shimada, H. & Sigiura, M. (1984) 'Sequence analysis of the junctions between a large inverted repeat and single-copy regions in tobacco chloroplast DNA', *Mol. Gen. Genet.* 194, 200-205

Tassopulu, D. & Kung, S.D. (1984) '*Nicotiana* chloroplast genome', *Theor. Appl. Genet.* 67, 185-193

Tewari, K.K. & Wildman, S.C. (1968) 'Function of chloroplast DNA. 1. Hybridisation studies involving nuclear and chloroplast DNA with RNA from cytoplasmic (80S) and chloroplast (70S) ribosomes', *Proc. Natl. Acad. Sci. USA* 59, 569-578

Tewari, K.K. (1971) 'Genetic autonomy of extra-nuclear organelles', *Ann. Rev. Plant Physiol.* 22, 141-168

Thinh, L-V. (1978) 'Photosynthetic lamellae of *Prochloron* (Prochlorophyta) associated with the ascidian *Diplosoma virens* (Hartmeyer) in the vicinity of Townsville', *Aust. J. Bot.* 26, 617-620

Thomas, P. (1983) 'Hybridisation of denatured RNA transferred or dotted to nitrocellulose paper', *Methods in Enzymol.* 100, 255-266

Thompson, W.F., Kaufman, L.S. & Watson, J.C. (1986) 'Induction of plant gene expression by light', *BioEssays* 3, 153-159

Thomson , J.A. (1980) 'Apparent identity of chromoplast and chloroplast DNA in daffodil, *Narcissus pseudonarcissus*', *Z. Naturforsch. Teil C.* 35, 1101-1103

Thomson, W.W. & Whatley, J.M. (1980) 'Development of non-green plastids', *Ann. Rev. Plant Physiol.* 31, 375-394

Thorne, S.W., Newcomb, E.H. & Osmond, C.B. (1977) 'Identification of chlorophyll b in extracts of prokaryotic algae by fluorescence spectroscopy', *Proc. Natl. Acad. Sci. USA* 74, 575-578

Tymms, M.J., Scott, N.S. & Possingham, J.V. (1983) 'DNA content of *Beta vulgaris* chloroplasts during leaf cell expansion', *Plant Physiol.* 71, 785-788

Uzzell, T. & Spolsky, C. (1974) 'Mitochondria and plastids as symbionts: a rival of special creation?', *Am. Scientist* 62, 334-343

van den Boogarrt, P., Samallo, J. & Agsteribbe, E. (1982) 'Similar genes for a mitochondrial ATPase subunit in the nuclear and mitochondrial genomes of *Neurospora crassa*', *Nature* 298, 187-189

van Ee, J.H., Vos, Y.J. & Planta, R.J. (1980) 'Physical map of chloroplast DNA of *Spirodela oligorrhiza*; analysis by the restriction endonucleases Pst I, XhoI and SacI', *Gene* 12, 191-200

Walbot, V. (1977) 'The dimorphic chloroplasts of the C_4 plant *Panicum maximum* contain identical genomes', *Cell* 11, 729-737

Watson, J.C. & Surzyaki, S. (1983) 'Both the chloroplast and nuclear genomes of *Chlamydomonas reinhardi* share homology with *Escherichia coli* genes for translational components', *Curr. Genet.* 7, 201-210

Whatley, J.M., John, P. & Whatley, F.R. (1979) 'From extracellular to intracellular: the establishment of mitochondria and chloroplasts', *Proc. R. Soc. Lond.* 204, 165-187

Willey, D.L., Howe, C.J., Auffret, A.D., Bowman, C.M. & Dyer, T.A. (1984) 'Location and nucleotide sequence of the gene for cytochrome f in wheat chloroplast DNA', *Mol. Gen. Genet.* 194, 416-422

Withers, N.W., Alberte, R.S., Lewin, R.A., Thornber, J.P., Britton, G. & Goodwin, T.W. (1978) 'Photosynthetic unit size, carotenoids and chlorophyll-protein composition of *Prochloron sp.*, a prokaryotic green alga', *Proc. Natl. Acad. Sci. USA* 75, 2301-2305

Wollgiegn, R. & Parthier, B. (1980) 'RNA and protein synthesis in plastid differentiation' in *Chloroplasts, Results and Problems in Cell Differentiation* (Reinert, J., Ed.), vol. 10, pp. 97-146, Springer-Verlag, Berlin

Wright, R.M. & Cummings, D.J. (1983) 'Integration of mitochondrial gene sequences within the nuclear genome during senescence in a fungus', *Nature* 302, 86-88

Ycas, M., Sugita, M. & Bensam, A. (1965) 'A model of cell size regulation' *J. Theoret. Biol.* 9, 444-470

Zhu, Y.S., Merkle-Lehman, D.L. & Kung, S.D. (1984) 'Light-induced transformation of amyloplasts into chloroplasts in potato tuber', *Plant Physiol.* 75, 142-145